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Review

Pathogenesis of murine cytomegalovirus infection

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Abstract

Infection of mice with murine cytomegalovirus (MCMV) is an established model for studying human cytomegalovirus (HCMV) infection. Similarly to HCMV infection, pathological changes and disease manifestations during MCMV infection are mainly dependent on the immune status of the mouse host. This review focuses mainly on the pathogenesis of MCMV infection in immunocompetent and immunodeficient and/or immature mice and discusses the principles of immunosurveillance of infection and the mechanisms by which this virus evades immune control.

Keywords: Murine cytomegalovirus; Pathogenesis; Immunosurveillance; Immune evasion

1. Introduction

Murine cytomegalovirus (MCMV) belongs to the genus cytomegalovirus (CMV) of the β-Herpesvirinae subfamily of the Herpesviridae family. CMVs are marked by strict species specificity, tropism for hematopoietic tissue and secretory glands, and a slow replication cycle. After the resolution of acute infection, CMVs establish persistent life-long infections characterized by alternate stages of virus productivity and latency. Although producing up to 200 potentially antigenic proteins during its sequential immediate early (IE), early (E) and late (L) phases of gene expression, transmission to a new host is achieved in the face of repeatedly primed antiviral immune responses.

The routes used by MCMV to enter the host have not yet been clearly determined. Infectious virus can easily be detected in saliva of chronically infected mice, and productive infection is confined to glandular epithelial cells of salivary glands for a prolonged period of time, even in a fully immunocompetent host. Therefore, it is likely that the saliva is the major source of virus for horizontal spread, and the major route of entry for MCMV is the epithelium of the gastrointestinal and the upper respiratory tract. Per oral infection of newborn mice with MCMV leads to virus spread similar to that following intraperitoneal virus administration (S. Jonjic, unpublished), which confirms that MCMV can enter through the epithelium of the gastrointestinal and/or respiratory tract. In addition, sexual transmission of MCMV and viral entry through the epithelium of the genitourinary tract could be an important means of its horizontal spread. After entry into the host, the virus spreads haematologically to various organs and infects many different cell types, including epithelial and endothelial cells, myocytes, brown fat adipocytes, fibrocytes, macrophages and bone marrow (BM) stromal cells.

Since MCMV infection shares many features with human cytomegalovirus (HCMV) infection, the mouse model has been extensively used for studying the pathogenesis of acute, latent and recurrent virus infections. Disease manifestations during MCMV infection can vary significantly, depending on the source of the virus (tissue culture (TC)- vs. salivary gland-derived virus (SGV)), the dose of virus and the route of inoculation, immune status of the mouse, the age at inoculation and the genetic background of the mouse. In this review, we will focus on several aspects of MCMV pathogenesis, including the immunosurveillance of infection and the mechanisms by which MCMV evades immune control.

2. MCMV infection in immunocompetent mice

Intraperitoneal or footpad injection of adult immunocompetent mice with TC MCMV (10^5 PFU) is usually asymptomatic, and it is not associated with serious damage to organ systems. This is consistent with the fact that very little infectious virus can be detected in tissues of infected mice, with

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the exception of salivary glands, which support prolonged virus replication. On the other hand, injection of a similar dose of SGV results in damage to multiple organs and tissues and high mortality even in fully immunocompetent hosts. SGV is produced as a homogenate of salivary glands from infected mice, the cellular components of which presumably include cytokines and hormones. These components may in part account for some of the virulent manifestations of SGV infection as compared to TC MCMV. It must be noted that just a single passage of SGV through cell culture leads to the loss of virulence. Interestingly, virus derived from other tissues ex vivo is not as virulent as SGV, indicating that this epigenetic phenomenon may be specific to virus grown in glandular epithelial cells of salivary glands.

The primary features of acute infection with SGV include high levels of virus replication in the spleen and the liver, with loss of the liver function [1,2] and immunosuppression [3,4]. We have shown that both histopathological changes and perturbation of the liver function correlate with the level of virus replication in this organ, stressing the important role of direct viral damage of hepatocytes in the pathogenesis of disease [5]. However, the global and confluent destruction of the liver observed during lethal infections is associated with some histopathological manifestations typically linked with high levels of circulating tumor necrosis factor-α (TNF-α). SGV infection also induces shock-like syndrome in infected mice, characterized by massive induction of cytokines late in infection [5]. Therefore, bystander effects related to the cytokines or other soluble mediators, in addition to direct damage caused by the virus replication, can contribute to liver disease and a fatal outcome of infection. The extent of damage to the spleen and suppression of immune responses appears to be closely linked with the degree of liver dysfunction, and ultimately, the death of infected mice.

3. Pathogenesis of MCMV in immunocompromised and immature mice

CMVs in general cause asymptomatic infection in immunocompetent hosts but are important opportunistic viruses affecting immunodeficient hosts. For example, HCMV infection is the leading opportunistic infection among AIDS patients and leads to variable clinical manifestations including pneumonitis, hepatitis, retinitis, esophagitis, colitis, and enchephalitis (reviewed in [6]). Similarly, infection of immunocompromised mice with TC MCMV is associated with a high level of virus replication and multiple organ damage, resulting in high morbidity and mortality [7]. Pneumonitis is a serious manifestation of HCMV infection, often fatal in immunosuppressed allogeneic transplant recipients (reviewed in [6]). MCMV infection of mice after sublethal γ-irradiation also causes interstitial pneumonia characterized by cell swelling and interstitial fluid exudation. Intracellular inclusions and active virus replication are observed in interstitial cells, pneumocytes, and endothelial cells of the lung [7]. Adrenal necrosis caused by HCMV has been described as a life-threatening pathophysiological manifestation among AIDS patients [8]. Similarly, MCMV infection of severely immunocompromised BALB/c mice induces focal necroses throughout the adrenal cortex [9]. Moreover, adrenalectomized BALB/c mice succumb to doses of virus up to fivefold lower than normally tolerated, indicating that an adrenocortical response is critical to survival during the early days after infection [10]. However, the extent to which lesions of adrenal glands contribute to high mortality of MCMV-infected immunodeficient mice and immunocompetent mice infected with SGV remains to be determined.

Reddehase and colleagues demonstrated that MCMV infection of immunodeficient mice causes BM aplasia [11]. MCMV-induced changes in the BM occur at the early stage of hematopoietic differentiation, at or before the renewal of the stem cell factor receptor-positive (SCF-R$^+$) stem and progenitor cells [12]. The antihematopoietic effect, however, could not be attributed to a direct effect of MCMV on the stem or progenitor cells, because progenitor cells derived from infected BM proliferate and differentiate normally after a rescue transfer to a healthy stromal microenvironment [11,13]. Since the proliferation of progenitor cells can be limited by a lack of support by stromal cells due to the MCMV infection, it appears that the stromal cells are the principal targets of MCMV infection. However, MCMV infection of BM is not associated with extensive cytolytic infection of stromal cells. Instead, the functional integrity of the stroma is impaired, including a reduced expression of essential hemopoietins such as SCF, granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6) [12,14].

HCMV infection is implicated as a potential contributor in atherosclerosis. BALB/c mice inoculated with MCMV express viral antigens in the endothelial and smooth muscle cells of the aortic wall. Similarly to early atherosclerotic lesions in humans, the accumulation of inflammatory cells in the aortic lumen co-localizes with the site of viral antigen expression [15]. The level of low-density lipoprotein cholesterol (LDL-C), the major lipid contributor to atherosclerotic plaques, is significantly increased in the serum of MCMV-infected mice, suggesting that MCMV infection contributes to atherosclerosis by immune injury and increased level of LDL-C in the serum [15]. In addition, MCMV infection induces myocarditis, characterized by a mononuclear cell infiltration, which varies from focal to intense inflammation with associated necrosis of myofibres and cytomegalic viral inclusion-bearing cells during acute phase of the infection [16]. The heart-infiltrating cells are predominantly CD8$^+$ T lymphocytes but also CD4$^+$ T cells, macrophages, B cells and neutrophils [17]. Antiviral antibodies that cross-react with cardiac myosin are produced during MCMV infection, suggesting the possibility that molecular mimicry contributes to the pathogenesis of autoimmune myocarditis following the infection [18].

The mouse model of CMV retinitis with features resembling those observed in human patients has been established [19]. MCMV inoculation via supraciliary route results in

infection of retinal glial cells and apoptosis of retinal neurons in spite of the fact that neurons are not infected. It has recently been shown that loss of the perforin cytotoxic pathway predisposes mice to MCMV retinitis [20].

3.1. MCMV infection in neonatal mice

HCMV infections are the leading cause of morbidity, mortality and mental retardation in congenitally and perinatally infected infants (reviewed in [21]). HCMV can be transmitted transplacentally from mother to fetus during pregnancy, during birth or through breast-feeding. Vertical transmission of MCMV is different from HCMV in that while it can be transmitted to newborn mice during birth or afterwards by breast-feeding and saliva, it cannot be transmitted transplacentally. Thus, MCMV is not an optimal model for congenital HCMV infection. Nevertheless, MCMV infection of neonatal mice recapitulates many clinical, pathohistological, and pathogenetic features of congenital HCMV infections [22]. Unlike adult mice acutely infected with TC MCMV, in which productive infection is terminated within 3–4 weeks, newborn mice that survive MCMV infection establish a long-lasting persistence in salivary glands and shed the virus in saliva for several months before termination of productive infection and establishment of latency [23]. Intraperitoneal infection of neonatal BALB/c mice with 1000 PFU of TC MCMV results in significant morbidity and mortality, causing disease that in many aspects resembles the infection of adult mice with more virulent SGV [24]. Immunohistological staining for viral IE antigen showed positive cells in a wide range of tissues and organs (Fig. 1). Confluent necroses were observed in the liver. The lesions were characterized by large patches of nuclear debris, coagulative necrosis and changes consistent with hypoxic necrosis. High levels of TNF-α induced in MCMV-infected neonatal mice may account for the observed severe liver damage and high frequency of mortality [24].

In contrast to adult mice, MCMV infection in newborn mice is characterized by virus dissemination within the central nervous system (CNS), associated with inflammatory changes and developmental abnormalities ([24] and S. Jonjic, unpublished). Immunohistological studies have revealed scattered CMV-positive cells throughout the brain, including the hippocampal region, the brain cortex, the ependymal lining of the lateral ventricles and the cerebellum. Histopathological studies have shown that inflammatory lesions develop from minimal changes during early days post-infection into significant inflammatory lesions at later times of infection. These lesions tend to increase in severity and are still pronounced at the 21st day post-infection, a time at which infectious virus is undetectable in the brain. These findings strongly suggest that the mechanisms responsible for virus clearance in the CNS are also responsible for development of late inflammatory lesions, and subsequent brain damage. However, multiple foci of necrosis not associated with lymphocytic cellular infiltrates are noted as well. The observed differences in the MCMV infection of brain between neonatal and adult mice can be explained not only by the immunological immaturity of neonatal mice, but probably also by the fact that in neonatal mice many cell types in the brain are in a stage of differentiation that is permissive for MCMV infection [25]. A still permeable, blood–brain barrier in neonatal mice is also likely to contribute to the hematological spread of the virus to the brain tissue. However, virus may gain access to the CNS by multiple routes. Cells expressing viral antigens can also be detected in the olfactory epithelium of infected animals [24], suggesting that MCMV can enter the brain of newborn mice via sensory epithelium.

Virus-associated damage in the newborn mouse extends beyond hepatic, neuronal and lymphatic tissues. Necrosis and inflammation is observed throughout the dermis of the skin. Viral antigens also can be detected in diverse cell types such as cells of hair follicles and the BM. Significant myositis and myocarditis also develop, as well as coagulative necrosis in the smooth muscle wall of the ureter [24]. MCMV also infects the endothelial and myointimal cells of the coronary arteries in newborn BALB/c mice [22], suggesting the possible association between perinatal infection and atherosclerosis. Interestingly, although intestinal epithelium has been described as a target for HCMV infection in immunodeficient humans, we failed to detect MCMV in intestinal epithelial cells of newborn mice.

4. Imunosurveillance of MCMV infection

Immune control of CMV infection is organized in a hierarchical and redundant manner by distinct elements of the immune system. Cellular immunity and interferons have been demonstrated to play a prominent role in control of primary MCMV infection, establishment of latency and prevention of productive virus reactivation (reviewed in [26,27]).

4.1. Early susceptibility of mice to MCMV infection correlates with their ability to mount NK cell response

The deficiency of NK cells in humans is rare, but such individuals suffer from unusually severe HCMV infection [28]. The importance of NK cells in the control of MCMV infection has been shown for several mouse strains, particularly for athymic nude mice and mice with severe combined immunodeficiency (SCID) [29]. Based on their susceptibility to MCMV infection during the early post-infection period, conventional mouse strains can be divided into two groups: MCMV-susceptible strains, represented by BALB/c mice, and MCMV-resistant strains, represented by C57BL/6 mice (reviewed in [30]). Resistance to MCMV strongly correlates with the ability of mouse strains to mount an effective NK cell response (Fig. 2A, B) controlled by the *Cmv1* locus, positioned inside the NK gene complex on mouse chromosome 6. The alleles of the *Cmv1* locus can either confer susceptibility (*Cmv1*^{s}, a recessive allele) or resistance (*Cmv1*^{r}, a dominant allele) to MCMV. The latter allelic form

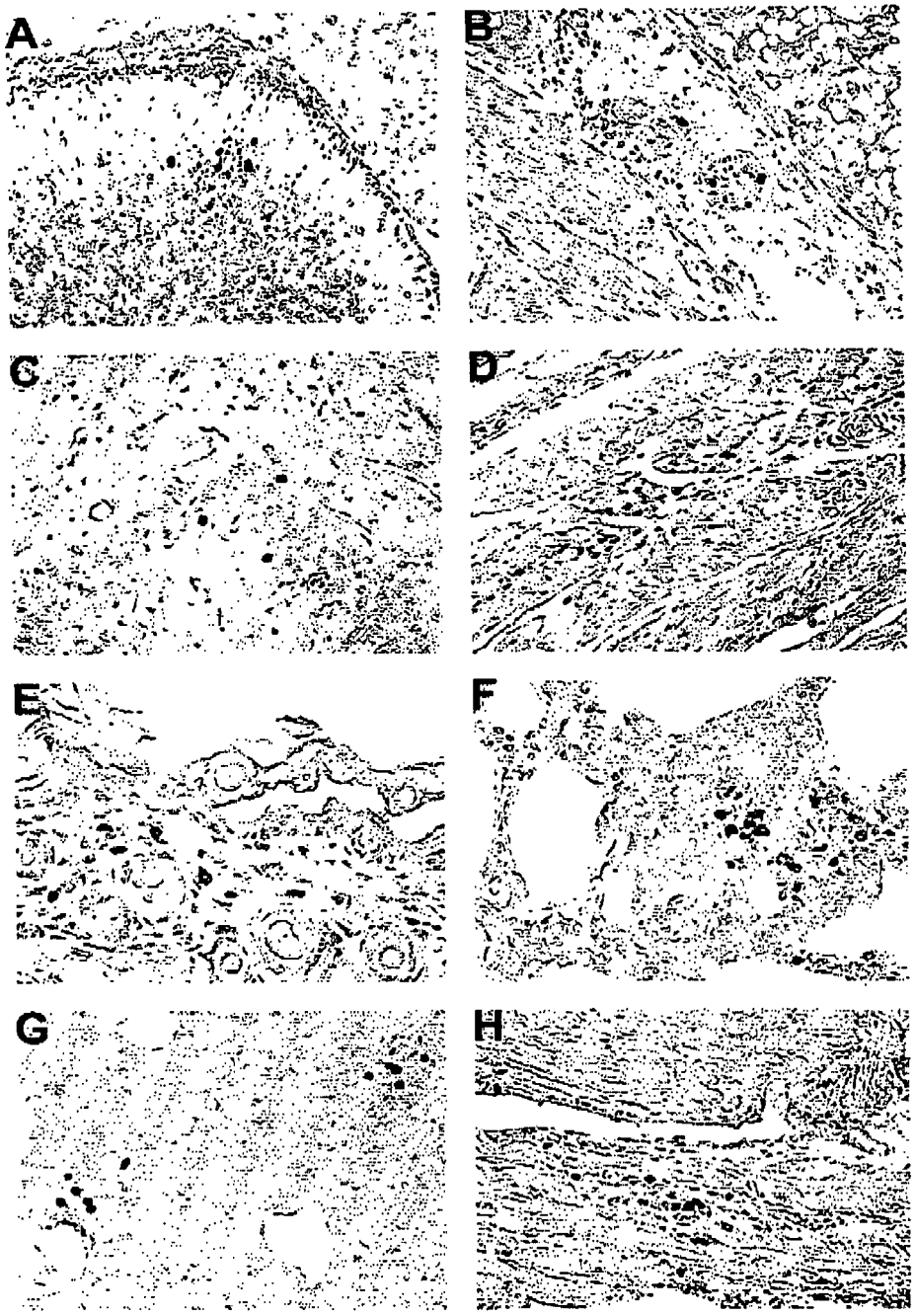

Fig. 1. Multiple organs and tissues of newborn mice can be infected by MCMV. Newborn mice were injected with 100 PFU of tissue-culture-grown MCMV, and their organs were analyzed for expression of MCMV pp89 IE protein recognized by CROMA 101 mAb. Paraffin sections were processed and stained, as described previously [5]. Magnification of the photomicrographs is 20×. Shown are: A, cerebellum; B, ganglion; C, hippocampus; D, skeletal muscle; E, dermis; F, lungs; G, liver; H, heart muscle.

restricts early MCMV replication in the spleen of resistant strains [31,32]. Recently, the *Ly49h* gene has been identified as the gene that mediates the *Cmv1* phenotype [33–35]. Depletion of NK1.1$^+$ or Ly49H$^+$ NK cells abolishes resistance to MCMV in C57BL/6 mice [34–36]. Ly49H binds specifically to a protein encoded by the MCMV *m157* gene, which has a structural homology to MHC class I molecules [37,38]. The fact that the product of the *m157* gene is the ligand for the activating NK cell receptor could be counterproductive from the virus point of view, and it is unclear why

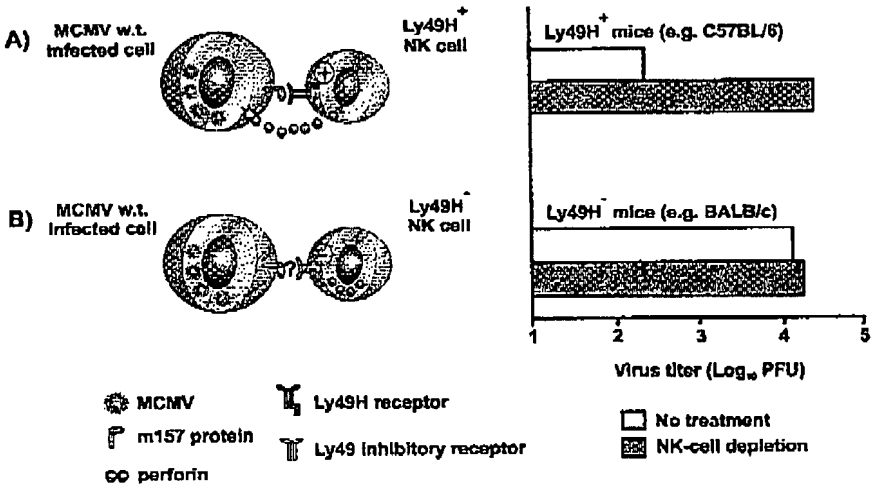

Fig. 2. Host and viral factors determine the susceptibility to MCMV infection. Natural resistance to MCMV is determined by expression of the activating NK cell receptor, Ly49H. (A) Activation of NK cells in C57BL/6 mouse strain is a consequence of binding of the Ly49H NK cell receptor to the MCMV *m157* gene product, leading to efficacious virus control in vivo, which can be compromised by depletion of NK cells. (B) In the BALB/c mouse strain, however, the absence of Ly49H NK cell receptor prevents NK cell activation and virus control. Adapted from *Nature Immunology* 3 (2002) 529–535, with copyright permission of The Nature Publishing Group [39].

MCMV has maintained the *m157* gene in its genome in spite of immunological selective pressure. However, since the product of *m157* also binds to the 129/J strain allelic form of Ly49I, an inhibitory NK receptor, it has been proposed that m157 might have originally evolved to serve as an immune evasion protein that delivers inhibitory signals to the NK cell subsets expressing Ly49I or Ly49I-like receptors [37]. Bearing in mind that Ly49H-negative mouse strains possess other activating NK receptors, such as NKG2D, it was unclear why these mouse strains are unable to mount an effective NK cell response. We have recently explained this paradox [39] by showing that MCMV inhibits NKG2D activation by down-regulation of its cognate ligands on infected cells (see Fig. 8 and paragraph on MCMV-mediated modulation of immune control).

4.2. Selective role of T cell subsets in the control of MCMV infection

Although NK cells play an important role during the early post-infection period in MCMV-resistant mouse strains, T lymphocytes are required for the termination of the productive infection and the establishment of latency. Originally, the protective capacity of T cells against MCMV infection was determined by adoptive transfer of MCMV-primed T cells into syngeneic sublethally γ-irradiated hosts [7,9]. These studies have shown that T lymphocytes obtained from draining lymph nodes of acutely infected immunocompetent mice, as well as memory T cells derived from latently infected mice, can prevent mortality and limit the virus replication in various organs and tissues. Furthermore, it has been shown that this antiviral effect of T lymphocytes was a function of the $CD8^+$ T cell subset, whereas $CD4^+$ T lymphocytes were inefficient and not required for immunocytotherapy mediated by $CD8^+$ T cells (Fig. 3). This protective function has been demonstrated not only by transfer of polyclonal T cell population but also by T cell lines of defined peptide specificities [40–42]. Adoptively transferred MCMV-primed $CD8^+$ T lymphocytes effectively limit virus replication in tissues, prevent histopathological damage and, ultimately, protect infected immunocompromised recipients from a lethal outcome of infection. Moreover, activated $CD8^+$ T lymphocytes are effective not only in prophylaxis but also in therapy of manifested MCMV disease. The validity of this immunotherapy model has been proven by clinical application of T cell transfer as a specific preemptive cytoimmunotherapy of HCMV disease in immunocompromised humans after BM transplantation [43].

Memory T cells have a powerful protective role against lethal MCMV infection after transfer into syngeneic immunodeficient recipients [9]. Recent studies indicate that antiviral memory $CD8^+$ T cell response differs between readily eliminated viruses (e.g. lymphocytic choriomeningitis virus and influenza A virus) and viruses that establish chronic/latent infection with continuous challenge of immune response. In the case of readily eliminated viral infections, which terminate with entire virus and antigen clearance, a large initial expansion of specific effector $CD8^+$ T lymphocytes is followed by a contraction phase [44]. It has recently been shown that a small pool of memory $CD8^+$ T cells, derived from effector cells, with the quality of self-renewal and rapid recall to the antigen, remains after such a type of viral infection [45]. During persistent virus infections, however, T cells are under continuous antigen boost, and specific $CD8^+$ T cells are maintained at high levels [46].

A characteristic shaping of the T cell repertoire and immunodominance of certain viral peptides between acute re-

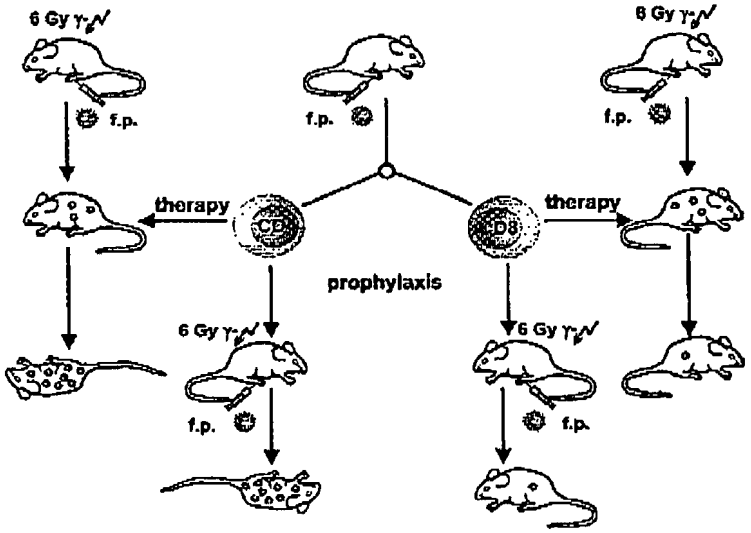

Fig. 3. CD8⁺ T cells are principal effector cells in the control of MCMV infection. BALB/c mice immunodepleted by total body γ-irradiation and injected with 10^5 PFU of MCMV develop multi-organ disease with high virus titre in their tissues and usually succumb to the infection. Prophylactic as well as therapeutic adoptive transfer of MCMV-primed syngeneic CD8⁺ T lymphocytes into infected and immunodepleted recipients leads to virus control and survival of infected animals. In contrast, CD4⁺ T cells derived from MCMV-primed immunocompetent donors fail to protect the recipient mice, and they frequently succumb to infection, similarly to mice that were not transferred with immune cells. f.p., footpad.

sponse and memory after MCMV infection was originally demonstrated by Reddehase and colleagues [40,41,47,48]. They have shown that memory CD8⁺ T cells specific for MCMV IE1 peptide accumulate in lymphoid tissue and as tissue-resident interstitial cells during latency, and that a majority of these cells show an activated ($CD62L^{low}$) phenotype [40,47]. Furthermore, these memory-effector T cells are protective against MCMV infection upon their adoptive transfer in vivo [40,48]. This finding is in accordance with the interpretation that IE1-specific CD8⁺ T cells are frequently resensitized during latent infection and may thus be involved in the maintenance of MCMV latency [47,49]. However, although this enrichment of memory T cells does not apply to T cells specific for three other MHC class I-restricted MCMV peptides (m04, m83 and m84), it is not exclusive for IE1 peptide either. Namely, CD8⁺ T cells specific for peptide derived from MCMV E protein m164 co-accumulate with IE1-specific CD8⁺ T cells in the latent effector-memory pool [40]. This pattern of memory kinetics has recently been reproduced in MCMV-infected mice by use of MHC class I tetramers loaded with MCMV immunodominant peptides [50]. Consistent with the explanation that accumulation of IE1-specific memory T cells is a consequence of frequent resensitization by the IE1 peptide during MCMV latency is the lack of accumulation of memory cells in mice after infection with recombinant vaccinia virus expressing MCMV IE1.

4.3. Plasticity of T cell response to MCMV infection

Since adoptive cell transfer experiments demonstrated that the essential effector cells responsible for virus clearance from tissues belong to CD8⁺ T lymphocyte subset, it could have been expected that mice devoid of CD8⁺ T lymphocytes would be unable to control MCMV infection. However, in contrast to this assumption, mice depleted of CD8⁺ T cells by cytolytic monoclonal antibodies or CD8 or β2-microglobulin gene-knockout mice develop an efficient immune effector function and eliminate the virus from tissues with clearance kinetics similar to normal mice [51,52]. It appears that CD4⁺ T cells can take over antiviral activity in animal hosts devoid of the CD8⁺ T cell subset. Contrary to CD4⁺ T lymphocytes derived from MCMV-infected immunocompetent mice, that were neither protective on their own nor required for the protective function of CD8⁺ T lymphocytes (Fig. 3), virus-specific CD4⁺ T cells induced in CD8-deficient hosts exert a protective function when transferred into lethally infected recipients (Fig. 4) [52]. Yet, in addition to CD4⁺ T cells, accessory cells including macrophages are required for limiting the viral spread. Therefore, it can be concluded that the immune system can compensate for the lack of function of one of its effector arms. However, there are exceptions to this principle, and some functions of either CD4⁺ or CD8⁺ T cells cannot be mutually compensated. For instance, mice depleted of CD4⁺ T cells were unable to eliminate the virus from salivary glands despite a strong influx of CD8⁺ T cells into this organ. In these mice, virus is localized to the acinar glandular epithelial cells, and a long-term, high-level productive infection is established [53]. It can be concluded that, although CD8⁺ T lymphocytes represent the major protective principle in MCMV infection, CD4⁺ T cells are essential for virus elimination and in the prevention of horizontal transmission of infection. In contrast, CD4⁺ T cells fail to compensate for the loss of the CD8⁺ T cell function during immunoreconstitution after syngeneic BM transplantation in

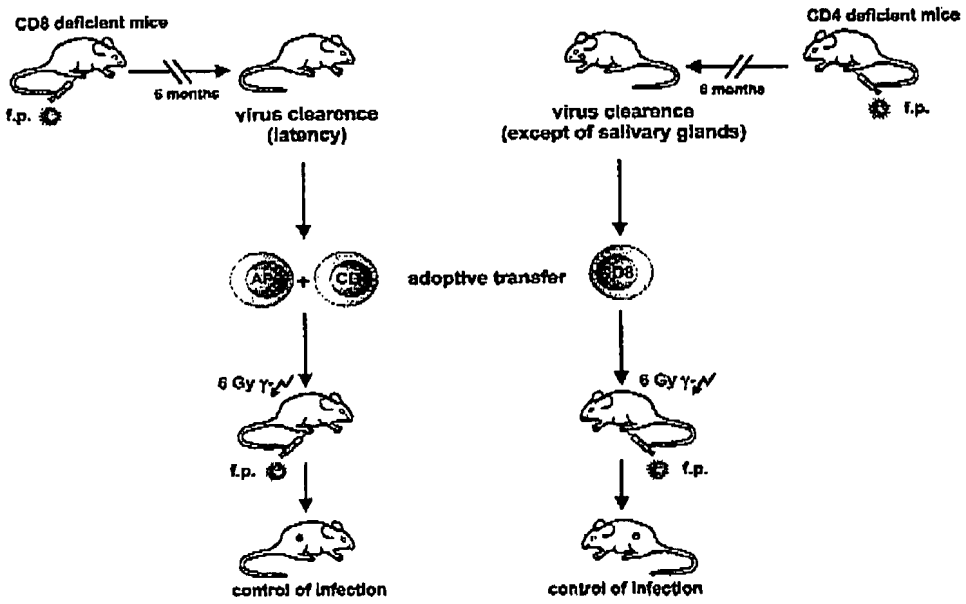

Fig. 4. Plasticity of T cell-mediated immune response. Adult thymectomized mice, depleted of either CD4+ or CD8+ T cells by cytolytic mAbs and footpad infected with MCMV, survive infection and clear the virus with clearance kinetics similar to those of normal mice. However, CD4+ T cell-depleted mice fail to clear the virus in the salivary glands. Adoptive transfer of T cells derived from either CD4+ or CD8+ T cell-depleted and MCMV-infected mice demonstrated that CD4+ T cells are not needed for the generation of specific CD8+ T cells. Furthermore, in contrast to CD4+ T cells derived from fully immunocompetent mice, MCMV infection in CD8+ T cell-deficient mice resulted in generation of protective antiviral CD4+ T cells. f.p., footpad.

BALB/c mice [54,55]. In this model, the reconstitution of CD8+ T cells is crucial for the prevention of fatal multiple-organ MCMV disease. Depletion of CD8+ T cells, but not of CD4+ T cells, performed during the reconstitution phase after BM transplantation, abolished the development of protective antiviral immunity and led to an enhanced virus replication in the most relevant organs.

4.4. The role of IFNs and TNF-α during MCMV infection

It is well established that interferon-γ (IFN-γ) and TNF-α play an important role in fatal and nonfatal MCMV disease progression. The function of these cytokines during MCMV infection can vary from protective [56–59] to pathogenetic [5,60]. Depletion of IFN-γ and TNF-α by antibodies has determined the crucial role of these factors in the control of MCMV replication and effector functions of antiviral CD8+ T cells in vivo [56,59]. IFN-γ and TNF-α act synergistically to induce an antiviral state in the uninfected cell, which reduces the production of infectious virions and prolongs the replication cycle following MCMV infection [57]. This kind of effector function is extremely important for recovery from primary infection. The sources for both cytokines during infection are NK cells and both CD4+ and CD8+ T lymphocytes. In addition to the well-documented antiviral effects of these cytokines, IFN-γ could have an important function in combating inhibitory viral effects on the antigen-presentation machinery [56]. By increasing the MHC gene expression, IFN-γ counteracts the block in antigen presentation that is mediated by products of viral immunomodulatory genes. Yet, IFN-γ has very little effect on cells that are already infected, and pre-treatment for at least 12 h prior to infection is required to derive a beneficial effect [56]. Thus, IFN-γ and TNF-α are important both at an early stage after infection when they prevent the radial transmission of the virus, and at a later stage, when they preserve the presentation of peptide-loaded MHC class I molecules to CD8+ T cells. However, the inability of infected cells to respond to IFNs suggests that MCMV possess a mechanism to interfere with IFN-induced gene transcription [56,57,61]. It has been shown that MCMV inhibits IFN-γ-induced MHC class II expression on macrophages at a stage subsequent to STAT1 activation and nuclear translocation [62], although the MCMV gene responsible for this viral evasion mechanism is still unknown. Attenuation of innate antiviral defenses appears to be a critical step in the life cycle of the CMVs, as evidenced by the finding that both HCMV and MCMV have evolved mechanisms to disrupt the JAK/STAT pathway of interferon signal transduction [63].

The significance of IFN-γ and TNF-α in MCMV infection has been demonstrated in vivo as well. Similarly to the depletion of CD4+ T cell subset, the neutralization of IFN-γ in vivo abolishes the antiviral activity of the CD4+ subset in the salivary glands, suggesting that CD4+ T cells mediate their antiviral effect by releasing IFN-γ [53,59]. Furthermore, IFN-γ is required for the antiviral activity of CTLs in long-term CD4-depleted mice [56]. IFN-γ released by NK cells is considered to play an important role in control of MCMV replication in liver [64]. Although TNF-α may play a detrimental role during lethal MCMV infection [5], similar

to IFN-γ, the neutralization of endogenous TNF-α also reduces the antiviral activity of $CD4^+$ T cells in immunocompetent as well as in $CD8^+$ subset-deficient mice [58]. The depletion of endogenous TNF-α in adoptive cell transfer recipients diminishes the antiviral function of $CD8^+$ T lymphocytes.

IFN-α/β play an essential role in limiting virus replication during the early stage after infection as well. These cytokines are produced by a subset of dendritic cells, which are induced during early MCMV infection and play an important role in activation of NK cells but also specific $CD8^+$ T cells [65]. It appears that plasmacytoid dendritic cells predominantly produce these innate cytokines. IFN-α receptor-deficient mice ($IFN\text{-}\alpha R^{-/-}$) are even more susceptible to MCMV than IFN-γ receptor-deficient mice ($IFN\text{-}\gamma R^{-/-}$), dying from 100-fold lower doses of virus [66,67]. In unpublished studies, we found that IFN-α and -β deficient mice are considerably more sensitive to infection by SGV than age-matched control mice (S. Jonjic, unpublished).

4.5. Immunosurveillance of latent and recurrent MCMV infection

Latency is usually defined as a nonproductive persistent infection in which the viral genome is present, but gene expression is restricted to a small subset of genes, and infectious virus cannot be detected. It has long been a dilemma to clarify whether the true molecular latency can be established at the level of the entire organism or if there is continuous but low-level virus production in face of a fully primed immune response. Highly sensitive assays of infectivity and RT-PCR analysis for detecting viral transcripts during latency have confirmed that molecular latency can be maintained at the level of the entire organism [68–70]. Kurz and Reddehase [49] have shown that in the lungs of latently infected mice the immediate early transcription unit *ie-1-ie-3* is active and generates *ie-1*-specific transcripts, but *ie-3* mRNA encoding the IE-3 transactivator, essential for early gene expression, is not detectable. Consequently, the transcription of E/L genes does not occur. The results accumulated so far indicate that reactivation is regulated at several checkpoints before the assembly and release of infectious virions: first, at the IE promoter/enhancer, also after IE1/3 transcription initiation, putatively at the step of IE1/3 precursor splicing or splice product stabilization. The possibility that additional control checkpoints exist is very likely [49,71].

Latent HCMV DNA has been detected in the $CD34^+$ myeloid progenitor cells, suggesting BM as a site of latency and source of latent virus at extramedullary sites [72–74]. However, keeping in mind that during primary MCMV infection viral DNA is cleared from BM and later, also from intravascular leukocytes, but retained in many organs in which MCMV establishes latency (lungs, liver, spleen, suprarenal glands, salivary glands and kidney), it is unlikely that BM serves as a prominent source of latent MCMV [23,69,75,76]. The cell type(s) harboring latent MCMV genome is still not clearly defined, although it is obvious that this cell(s) must be widely distributed. Some authors have suggested hystiocytes and endothelial-like cells, stromal cells of the spleen and macrophages/dendritic cells and their progenitors as cellular sites of MCMV latency (reviewed in [77]).

Although early studies indicated a protective role of administered antibodies during acute infection, studies on B cell-deficient mice demonstrated that antibodies are not essential for the resolution of the primary infection [68,78]. These mice clear the virus and establish viral latency with clearance kinetics indistinguishable from normal mice. The load of the latent viral genome, which represents an independent risk factor for recurrent infection [23], is also identical between B cell-deficient and normal mice [78]. The reactivation from latency is the only stage at which the absence of antibodies alters the phenotype of MCMV infection, since depletion of cellular immune control in B cell-deficient mice results in higher virus titre compared with seropositive control animals [68]. Therefore, we took the advantage of the enhanced ability to detect recurrent MCMV in B cell-deficient mice to study the selective role of $CD8^+$ and $CD4^+$ T cells, NK cells and cytokines in surveillance of the recurrent CMV infection (Fig. 5) [68]. The stepwise withdrawal of immune effector functions revealed that the $CD8^+$ and $CD4^+$ T cell subsets, as well as NK cells, contribute in a hierarchical and redundant fashion to restrict MCMV recurrent infection. IFN-γ also participates in the control of MCMV recurrence, and its neutralization, when combined with the depletion of either $CD8^+$ or $CD4^+$ T lymphocytes, gives rise to recurrent infection. Thus, from a clinical perspective,

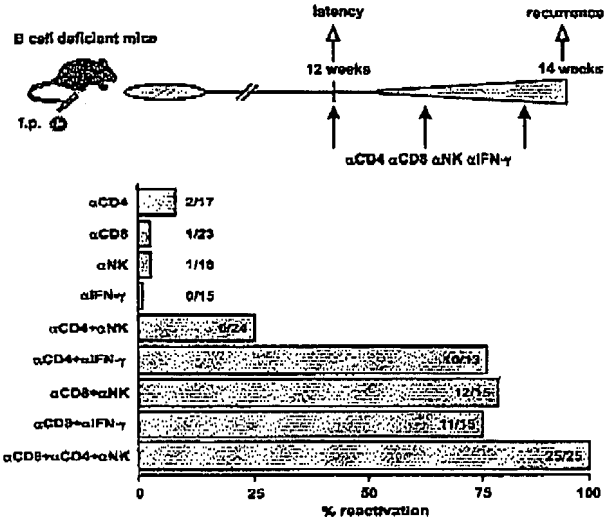

Fig. 5. Hierarchical and redundant mechanisms of immune control of latent MCMV infection. Latently MCMV-infected B cell-deficient mice were depleted of $CD4^+$ T cells, $CD8^+$ T cells, NK cells and IFN-γ alone or in different combinations as indicated. Two weeks later, organs (salivary glands, lungs and spleen) were analyzed for infectious MCMV. Mice showing recurrent virus in any tested organs were scored positive. Adapted from the *Journal of Experimental Medicine* 188 (1998) 1047–1054, with copyright permission of The Rockefeller University Press [68]. f.p., footpad.

CMV latency is tightly controlled by the immune system, and the reactivation from latency, and subsequent serious morbidity and mortality occurs whenever the essential components of immune control mechanisms are compromised (e.g. in immunocompromised transplant patients). It appears that MCMV initiates the productive replication cycles immediately and with a high frequency, resulting in rapid recurrence in the absence of appropriate immune control. However, one has to consider the possibility that high frequency of reactivation in our model should not necessarily be only a consequence of depletion of immune cells, but may also be induced by cytokines and other factors released due to cell depletion in vivo. It has been suggested that the inflammatory immune response to allogeneic transplantation could be the first step in the reactivation of the virus in transplant recipients (reviewed in [79]). Accordingly, some studies also indicate that the proinflammatory cytokines may play a role in CMV reactivation from latency [80].

5. MCMV-mediated modulation of immune control

5.1. MCMV evasion from CD8$^+$ T cells

Viruses have "studied" immunology over millions of years of coevolution with their hosts, and therefore, it is not surprising that MCMV, similarly to HCMV, encodes a number of proteins aimed at ruining the effectiveness of the immune response of their hosts (reviewed in [81]). However, in spite of its many immunoevasion strategies, this virus is harmless for immunocompetent hosts, and it is still not clear why CMVs have evolved and maintained so many immunomodulatory functions. The most plausible theory would be that these immunoevasins help the virus to both establish latent infection and to reactivate and spread in face of the fully primed immune control. Since MHC class I-restricted CD8$^+$ T cells are the principal effectors during MCMV infection, it is understandable that the MHC class I antigen-presentation pathway is selected as a major target for immunoevasion. Although there is no sequence homology between the genes of HCMV and MCMV that encode proteins that modulate the expression of MHC class I molecules, both viruses effectively and redundantly inhibit class I antigen presentation through multiple gene functions (Fig. 6) (reviewed in [82]). Three MCMV gene products alter MHC class I function: m152, m04 and m06 (reviewed in [83]). The gene *m152*, a member of the *m145* gene family, encodes the type I glycoprotein of 40 kDa which retains assembled MHC class I complexes in the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and thereby prevents the presentation of MCMV peptides to CD8$^+$ T cells at an early stage of infection [84]. The deletion of the *m152* gene results in an attenuated virus with impaired pathogenicity and replication in vivo due to more stringent immune control by CD8$^+$ T lymphocytes. Importantly, this attenuation fails to manifest in mice deficient for antigen presentation via the MHC class

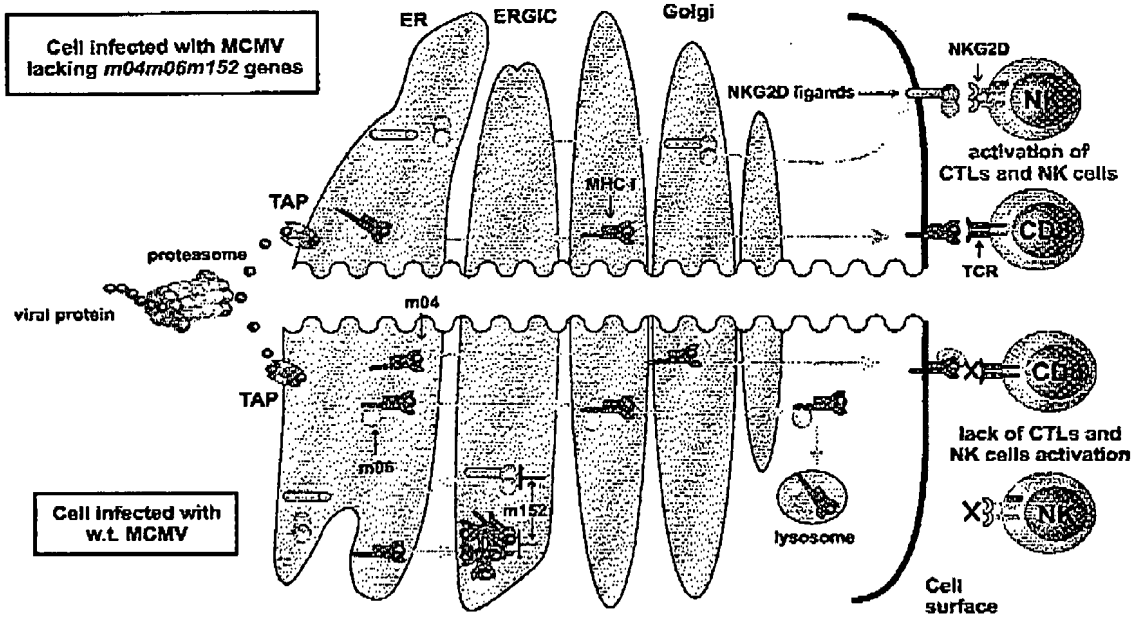

Fig. 6. MCMV evasion from CD8$^+$ T cells and NK cells in BALB/c mice. Upper panel: cell infected with an MCMV strain that lacks three major MHC class I evasion genes (*Δm04Δm06Δm152*) normally presents viral peptides in MHC class I complex molecules and stress-induced NKG2D ligands on its surface, leading to activation of CD8$^+$ T cells and NK cells. Lower panel: Cell infected with w.t. MCMV efficiently avoids immune control mediated by CD8$^+$ T and NK cells. The *m04* gene product binds to MHC class I in the ER and remains associated throughout its transport to the cell surface. The *m06* gene product binds to MHC class I molecules in the ER and redirects them to the lysosome. The *m152* gene product has a dual function. It retains MHC class I molecules in the ERGIC/*cis*-Golgi compartment and down-regulates NKG2D ligands.

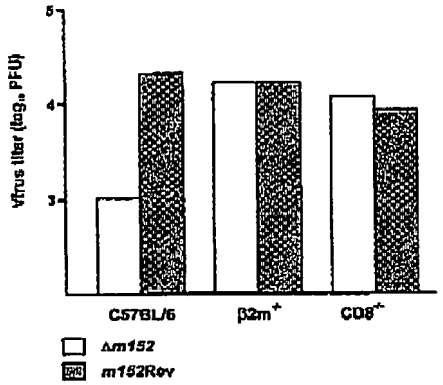

Fig. 7. Deletion of the MCMV gene *m152* sensitizes the virus for $CD8^+$ T cell control. C57BL/6, $\beta_2m^{-/-}$ and $CD8^{-/-}$ mice were infected with either *m152* deletion mutant (Δ*m152*) or its revertant virus (*m152*Rev), and virus titres in lungs were determined 10 days after infection. No difference between mutant and revertant viruses was observed in $\beta_2m^{-/-}$ and $CD8^{-/-}$ mice, whereas *m152* deletion mutant was clearly attenuated in C57BL/6 mice compared with *m152* revertant virus. Reproduced from the *Journal of Experimental Medicine* 190 (1999) 1285–1296, with copyright permission of The Rockefeller University Press [85].

I pathway (Fig. 7) [85]. The second MHC class I evasion function is encoded by the MCMV gene *m06*, which belongs to the *m02* gene family and encodes for a 48 kDa type I transmembrane glycoprotein. *m06*/gp48 binds to properly folded MHC class I molecules in the endoplasmic reticulum (ER) and redirects their transport to the endocytic route for rapid proteolytic degradation [86]. As a consequence, *m06*-expressing cells are not able to present antigens to $CD8^+$ T cells. The third MCMV gene which modulates the MHC class I pathway, *m04*, encodes for a 34 kDa type I transmembrane glycoprotein, which binds to MHC class I complexes in the ER, leaves the ER in association with MHC class I complexes and remains associated throughout their transport to the cell surface [87]. It has been speculated that *m04* could oppose the effect of *m152* by rescuing some class I molecules from retention, thereby preventing NK cell lysis of the infected cell, which can be activated by the loss of surface MHC class I molecules. The primary function of *m04* as a cytotoxic T lymphocyte (CTL) immune evasion gene has recently been demonstrated: it does not interfere with MHC class I transport but rather with the interaction between MHC class I and the T cell receptor [88]. It is likely that the evasion gene products supplement each other in disrupting antigen-presentation machinery. The expression of *m152*, but not of *m04*, is required to completely block antigen presentation to D^b-restricted CTL clones, but the expression of both *m152* and *m04* is necessary for the complete abrogation of antigen presentation to K^b-restricted CTL clones [88].

Using MCMV mutants with combined deletions of *m04*, *m06* and *m152* genes, Koszinowski and colleagues [89] have

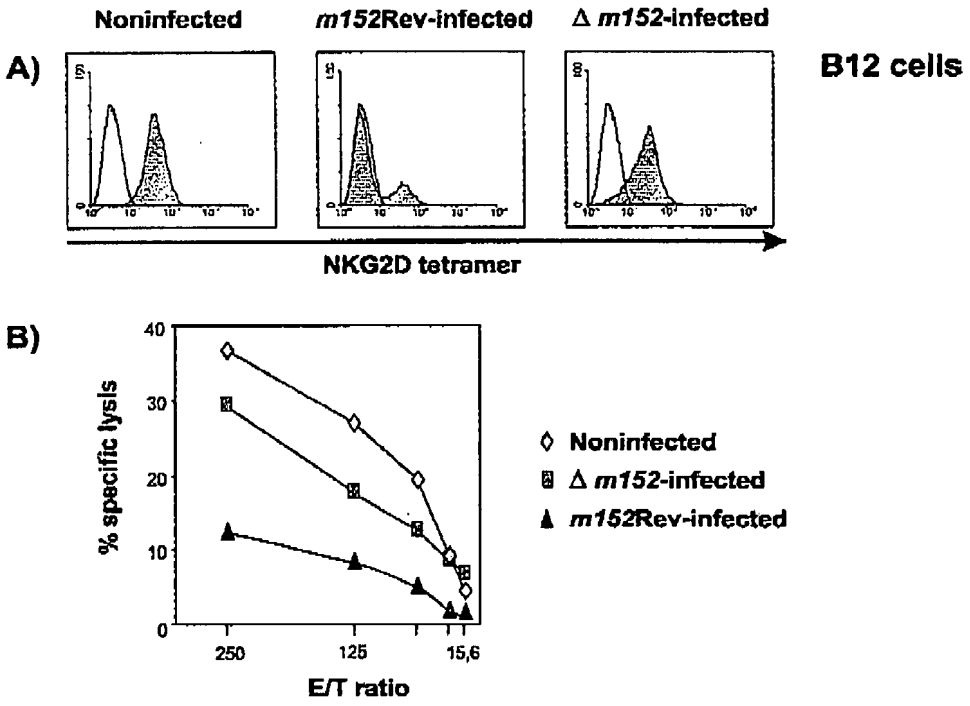

Fig. 8. The *m152*/gp40 down-regulates NKG2D ligands and prevents NK cell-mediated lysis of infected cells. (A) B12 cells (immortalized line of BALB/c fibroblasts) were infected with either *m152* deletion mutant (Δ*m152*) or *m152* revertant virus (*m152*Rev) and analyzed for expression of surface NKG2D ligands by staining with PE-labeled NKG2D tetramers 12 h after infection. (B) A 4-h ^{51}Cr-release assay was performed with NK cells from BALB/c mice as effectors and B12 cells infected with either Δ*m152* or *m152* revertant virus as targets. B12 cells infected with the *m152* revertant virus acquired resistance to NK cell lysis. Reproduced from *Nature Immunology* 3 (2002) 529–535, with copyright permission of The Nature Publishing Group [39].

recently shown that individual MHC class I alleles are differentially affected by immunoevasive genes and that the isolated deletion of *m06* has the strongest effect on MHC class I. They demonstrated that the *m06* and *m152* gene products cooperate in their function, but that in contrast, *m04* and *m152* gene products act antagonistically.

5.2. MCMV evasion from NK cells—dual function of MCMV m152/gp40

MHC class I molecules serve as a restriction principle in both adaptive and innate immune responses. These molecules interact with inhibitory receptors of NK cells and provide NK cells with sufficient negative signals to override the activating signals. How does the virus deal with the fact that down-regulation of MHC class I molecules to avoid CD8 recognition exposes infected cells to NK cell control? How can we explain the minimal contribution of NK cells in the control of MCMV replication observed in BALB/c and other *Cmv1*^{s} strains, which lack Ly49H, but possess other activating NK cell receptors, like NKG2D? We have recently shown that the inability of NK cells to act effectively against MCMV-infected cells in BALB/c mice is due to the down-regulation of ligands for NKG2D receptor by the viral *m152* gene product [39] (Fig. 8A). Thus, NK cells of MCMV-sensitive mouse strains have the ability to react to MCMV-infected cells, but the *m152* gene product inhibits their activity. The deletion of the *m152* gene lifted this inhibition, rendering otherwise NK-resistant virus-infected cells susceptible to NK cell control (Fig. 8B). Murine NKG2D interacts with three cell surface ligands related to MHC class I molecules: Rae-1 (α, β, γ, δ and ε isoforms), H-60, and MULT-1 [90–92]. Based on the constitutive difference in expression of H-60 between BALB/c and C57BL/6 mice, which do and do not express H-60, respectively, it was concluded that *m152*/gp40 interferes with the expression of H-60. Yet, the possibility that differential expression of other NKG2D ligands between BALB/c and C57BL/6 strains is responsible for the lack of *m152*-mediated NK inhibition in C57BL/6 mice could not be excluded. Recently, Lodoen et al. [93] demonstrated that gp40 specifically down-regulates Rae-1 α, β, γ, δ and ε.

NKG2D is expressed not only on NK cells but also on activated macrophages and T lymphocytes, where it serves as a co-activating receptor [91,94,95]. Therefore, the biological functions of gp40 could have three consequences: (1) evasion of innate immune responses through activity on the NKG2D ligands; (2) interference with NKG2D ligands as accessory molecule functions and subsequent CTL functions; and (3) by its activity on MHC class I it down-modulates the TCR ligand directly.

An additional means by which the virus can inhibit NK cells is by encoding a homologue of MHC class I molecules. The MCMV genome encodes a homologue for MHC I, designated m144 [96]. It has been hypothesized that the m144 protein functions as a mimic of host MHC I molecules and acts as a decoy for NK cells by engaging inhibitory NK receptors, thereby protecting infected cells from NK cell-mediated lysis. Recombinant MCMV, in which the class I homologue gene has been disrupted, shows severely restricted replication during the acute stage of infection compared with wild type (w.t.) MCMV [96]. In vivo depletion of NK cells restored the virulence of the *m144* deletion mutant, confirming that *m144* contributes to immune evasion through interference with NK cell-mediated clearance. Expression of m144 on tumor cells also indicates that m144 has an inhibitory effect on NK cells [97]. However, since the receptor on NK cells for m144 remains unknown, the possibility that the m144 protein inhibits NK cells by a mechanism other than serving as ligand for inhibitory NK receptors cannot be excluded.

5.3. MCMV interference with other components of the host's immune system

MCMV interferes with other components of the host's immune system as well. Since chemokines are important during early antiviral inflammatory responses, it was presumed that viral homologues of chemokines and chemokine binding proteins may enable the virus to evade or disarm the normal host inflammatory response. MCMV encodes a CC chemokine homologue, MCK-2 (*m131/129*) [98]. In vivo studies have shown that expression of *m131/129* during acute infection contributes to an increase in the inflammatory response at the site of inoculation, higher peak levels of viremia, and more efficient dissemination of MCMV to the salivary glands [99]. Chemokines impart their biological activities through binding specific seven-transmembrane G-protein-coupled receptors, which are expressed on a variety of leukocytes. Upon ligand binding, chemokine receptors activate a series of signaling pathways involving heterotrimeric G proteins, intracellular Ca^{2+} release and other secondary messengers. The MCMV-encoded chemokine receptor, M33, also resembles cellular genes and may play a role in modulating the host response for the benefit of viral tropism, propagation and dissemination [100]. It has been shown that deletion of the *M33* gene of MCMV results in severe restriction of virus growth in the salivary glands, a major site of viral replication and transmission.

Another MCMV gene, *m138 (fcr-1)*, encodes for an 88 kDa glycoprotein located at the surface of infected cells which strongly binds the Fc fragment of murine IgG [101]. Hypothetically, viral proteins that directly interact with host immunoglobulins could have potential to interfere with humoral immunity by modifying the antibody response of the host. A *m138* deletion mutant virus exhibits reduced growth in various organs of infected mice, compared with w.t. MCMV. However, virus replication in antibody-deficient mice has demonstrated that the attenuating effect of the *fcr-1* deletion is not linked to its IgG-specific function [102].

Recently, the mechanism by which MCMV protects infected cells from destruction by homologous complement has been identified. By inducing expression of the membrane cofactor protein CD46, the regulator of complement activa-

tion, MCMV can prevent lysis of infected cells by mouse complement [103]. MCMV has also evolved mechanisms for inhibition of MHC class II expression (e.g. by interference with the IFN-γ signaling or by induction of IL-10) and thereby escapes immunosurveillance by $CD4^+$ T lymphocytes (reviewed in [104]), although the gene(s) responsible are still unknown.

Since apoptosis of infected cells is considered an efficient means of protection from infectious agents, many viruses encode gene products that actively inhibit apoptotic destruction. Products of two HCMV genes prevent apoptosis. The HCMV *UL36* gene encodes an inhibitor of caspase-8 activation [105], and the *UL37* gene encodes a viral mitochondrial inhibitor of apoptosis, which blocks mitochondrial megapore activation [106]. MCMV encodes a UL36 homologue that binds to the endogenous procaspase-8 [107]. The MCMV gene *m45*, which shows sequence homology to ribonucleotide reductase genes and governs endothelial cell tropism, prevents apoptosis in productively infected endothelial cells, suggesting its cell type specific role in preventing apoptosis [108]. Since endothelial cells play a role not only in virus dissemination and latency but also in vascular disease [67], decreased apoptosis of these cells could contribute to vascular disease by thickening of vascular wall.

6. Concluding remarks

MCMV infection is still the leading animal model to study pathogenesis of HCMV infection. Although there is an increase in understanding of different aspects of MCMV pathogenesis, many important questions still remain unanswered. Keeping in mind that the immunocompetent mouse host can easily control primary infection and bring the virus to a nonproductive latent stage, the primary function of so many viral immunoregulatory genes affecting almost every component of the immune response is still not clear. It remains to be tested whether these immunoregulatory genes are necessary for the virus to be maintained in the latent stage, from which reactivation can occur periodically in spite of a fully sensitized immune response. Another possibility would be that immunoregulatory viral genes are important to prevent immunopathological damage of the host during acute and chronic infection. We still need to learn more about the viral tropism, and which cells and which conditions allow virus replication in vivo. It is also not clear whether productive MCMV infection can be noncytopathic in some cell types, particularly those that are vital for the animal host. For instance, we now know that different cell types in CNS can be infected during the perinatal period, but the mechanisms of MCMV clearance and the pathogenetic consequences of infection and local immune response are completely unknown. Resolving these, among many other questions, could aid in the development of new strategies for the treatment of CMV infection. It is likely that the MCMV model for HCMV infection will continue to profit from studies using specific virus deletion mutants, combined with studies of these genes in isolation, as well as from the use of gene-knockout mice.

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Murine cytomegalovirus resistant to antivirals has genetic correlates with human cytomegalovirus

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Human cytomegalovirus (HCMV) resistance to antivirals is a significant clinical problem. Murine cytomegalovirus (MCMV) infection of mice is a well-described animal model for *in vivo* studies of CMV pathogenesis, although the mechanisms of MCMV antiviral susceptibility need elucidation. Mutants resistant to nucleoside analogues aciclovir, adefovir, cidofovir, ganciclovir, penciclovir and valaciclovir, and the pyrophosphate analogue foscarnet were generated by *in vitro* passage of MCMV (Smith) in increasing concentrations of antiviral. All MCMV antiviral resistant mutants contained DNA polymerase mutations identical or similar to HCMV DNA polymerase mutations known to confer antiviral resistance. Mapping of the mutations onto an MCMV DNA polymerase three-dimensional model generated using the *Thermococcus gorgonarius* Tgo polymerase crystal structure showed that the DNA polymerase mutations potentially confer resistance through changes in regions surrounding a catalytic aspartate triad. The ganciclovir-, penciclovir- and valaciclovir-resistant isolates also contained mutations within MCMV M97 identical or similar to recognized GCV-resistant mutations of HCMV UL97 protein kinase, and demonstrated cross-resistance to antivirals of the same class. This strongly suggests that MCMV M97 has a similar role to HCMV UL97 in the phosphorylation of nucleoside analogue antivirals. All MCMV mutants demonstrated replication-impaired phenotypes, with the lowest titre and plaque size observed for isolates containing mutations in both DNA polymerase and M97. These findings indicate DNA polymerase and protein kinase regions of potential importance for antiviral susceptibility and replication. The similarities between MCMV and HCMV mutations that arise under antiviral selective pressure increase the utility of MCMV as a model for *in vivo* studies of CMV antiviral resistance.

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INTRODUCTION

Human cytomegalovirus (HCMV) is an important pathogen of immunocompromised individuals, and the development of antiviral-resistant HCMV strains is an increasing hindrance to the successful treatment and prevention of CMV-related illness. Animal models are necessary for *in vivo* studies of CMV antiviral susceptibility and resistance due to the species-specificity of HCMV that restricts permissive replication to humans and human cell lines. Murine cytomegalovirus (MCMV) infection in mice is an excellent model that has been extensively studied with respect to pathology and immunology (Hudson, 1979; Koffron *et al.*, 1998; Lagenaur *et al.*, 1994; Yuhasz *et al.*, 1994). The entire MCMV genome has also been sequenced, and shares many regions of similarity with HCMV (Rawlinson *et al.*, 1993, 1996).

HCMV and MCMV are inhibited by antivirals that target the viral DNA polymerase, including nucleoside analogues aciclovir (ACV), adefovir (ADV), cidofovir (CDV), ganciclovir (GCV), penciclovir (PCV), the ACV pro-drug valaciclovir (VCV) and the pyrophosphate analogue foscarnet (PFA) (De Clercq, 2001; Rawlinson, 2001; Smee *et al.*, 1995). HCMV and MCMV are inhibited by similar concentrations of these antivirals *in vitro*, except MCMV exhibits increased sensitivity to ACV (Boyd *et al.*, 1993, Chrisp & Clissold, 1991; Cole & Balfour, 1987; Rawlinson *et al.*, 1997; Smee

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et al., 1995; Xiong *et al.*, 1997a, b). The nucleoside analogues ACV, ADV, CDV, GCV and PCV require intracellular phosphorylation prior to incorporation into replicating viral DNA by the HCMV DNA polymerase (De Clercq, 2001). In HCMV-infected cells, ACV, GCV and PCV are initially monophosphorylated by the HCMV UL97 protein kinase, and further phosphorylated to the triphosphate form by cellular enzymes (Biron *et al.*, 1985; Littler *et al.*, 1992; Sullivan *et al.*, 1992; Talarico *et al.*, 1999; Zimmerman *et al.*, 1997). ADV and CDV are monophosphorylated and do not require activation by viral proteins prior to cellular conversion to their triphosphate forms (Xiong *et al.*, 1997a, b).

HCMV strains resistant to ADV, CDV, PFA or GCV can develop through mutations of the DNA polymerase gene (encoded by UL54), which can confer multi-drug resistance depending on the mutation position (Chou, 1999; Chou *et al.*, 2003; Erice, 1999). HCMV UL97 protein kinase mutations are most often described as responsible for the development of GCV-resistance in HCMV isolates, although earlier studies omitted assessment of DNA polymerase mutations (Chou, 1999; Erice, 1999). Although currently not documented in clinical isolates, mutations of HCMV UL97 protein kinase can confer resistance to ACV and PCV (Talarico *et al.*, 1999; Zimmerman *et al.*, 1997), with potential implications for the use of VCV prophylaxis (Feinberg *et al.*, 1997; Lowance *et al.*, 1999).

MCMV M54 and M97 are positional, sequence and primary structure homologues of HCMV DNA polymerase and UL97 protein kinase (Elliott *et al.*, 1991; Rawlinson *et al.*, 1993, 1996). The MCMV homologues retain DNA polymerase and protein kinase domain regions important for protein function (Elliott *et al.*, 1991; Rawlinson *et al.*, 1997). No other protein kinase homologues are known to be encoded by MCMV (Rawlinson *et al.*, 1996). Phosphorylation assays have failed to show detectable levels of ACV- and GCV-phosphorylation either in MCMV-infected cells or by a recombinant M97 protein, despite MCMV susceptibility to ACV and GCV *in vitro* (Burns *et al.*, 1981; Ochiai *et al.*, 1992; Wagner *et al.*, 2000). However, while HCMV UL97 substituted for MCMV M97 enhances GCV phosphorylation by recombinant MCMV, HCMV UL97 does not complement the function of MCMV M97 with respect to virus replication (Wagner *et al.*, 2000). Additional correlative information is therefore necessary to determine the complementarities of HCMV and MCMV antiviral susceptibility and virus replication.

In order to further analyse CMV replication and antiviral susceptibility, we have generated a series of MCMV mutants resistant to ACV, ADV, CDV, GCV, PCV, PFA and VCV and characterized the genetic mutations associated with the resultant changes in phenotype. Regions of MCMV DNA polymerase and the putative protein kinase (pM97) important for MCMV antiviral susceptibility and virus replication have been identified. The mutations of these MCMV antiviral-resistant strains demonstrate remarkable similarity to mutations known to confer antiviral resistance to HCMV isolates defined from infected patients, and correlate with DNA polymerase and protein kinase regions of functional importance.

METHODS

Antivirals. Reagent grade ACV and VCV were kindly provided by GlaxoWellcome (UK), ADV and CDV by Gilead Sciences (USA), GCV by Roche Pharmaceuticals (Australia), PCV by SmithKline Beecham Pharmaceuticals (UK) and PFA by Astra Pharmaceuticals (Australia). Antivirals were resuspended in pyrogen-free water (Baxter) to a final concentration of 10 μM (except PFA, which was resuspended to a final concentration of 50 μM), filter-sterilized and stored in aliquots at −20 °C until use.

MCMV strains. MCMV laboratory strain Smith was obtained from ATCC. The virulent laboratory strain K181 and nine wild-type MCMV isolates (G3A, G4, K17A, K17G, N1, W2, W5, W8 and W9) were kindly provided by Professor Geoffrey Shellam from the Department of Microbiology, University of Western Australia, Perth, Australia. Viral titres were quantified in primary mouse embryo fibroblasts (MEFs) by standard plaque assays, as described previously (Scott *et al.*, 2000).

Generation of antiviral-resistant MCMV isolates. MCMV antiviral resistant mutants (ACVres, ADVres, CDVres, GCVres, PCVres, PFAres and VCVres) were generated by continuous passaging of MCMV laboratory strain Smith in MEFs in increasing concentrations of each antiviral. Mutants were selected against both ACV and the ACV pro-drug VCV, to compare the resistance mutations generated by antiviral agents that differed in bioavailability. Initially, Smith strain was cultured in 25 cm^2 flasks containing minimal essential media (MEM) + 2 % fetal bovine serum (FBS) and 0·1 μM of antiviral. Virus was passaged to new MEF cultures after 100 % cytopathic effect (CPE) was observed, or one week of culture where CPE was less than 100 %, and antiviral concentration increased twofold at each passage. At higher antiviral concentrations, cultures were carried out in six-well plates, where plates were centrifuged at 600 *g* for 30 min to enhance virus infectivity. Passaging of virus continued to the highest concentration of antiviral where virus continued to be isolated without cellular toxicity (Table 1).

Each antiviral-resistant mutant was plaque purified a total of five times by culturing serial 1 : 10 dilutions of virus and sterile selection of single plaques using a pipette set at 20 μl. Single plaques were amplified by culture in six-well plates containing MEFs between purification rounds. At the final round of plaque purification, mutant virus was cultured in the presence of media without antiviral and cell-associated virus harvested and stored in MEM + 10 % FBS at −80 °C.

Plaque reduction assays (PRA). PRA was carried out in triplicate using 50 p.f.u. per well of virus as described previously (Rawlinson *et al.*, 1997), except that plates were centrifuged at 600 *g* for 30 min following inoculation of virus. The concentration range for each antiviral was as follows: 0·47–120 μM ACV (increased to 1·825–480 μM for analysis of the VCV-resistant mutant), 0·93–240 μM ADV, 0·0625–16 μM CDV, 0·625–160 μM GCV, 1·25–320 μM PCV, 6·25–1800 μM PFA and 1·825–480 μM VCV. The 50 and 90 % inhibitory concentrations (IC_{50} and IC_{90}) were calculated by linear regression from plots of percentage reduction in plaque numbers at each antiviral concentration against log drug concentrations. Resistance was defined as a greater than twofold increase in IC_{50} and IC_{90} values compared with parent (Smith) strain.

M54 and M97 DNA PCR. M54 and M97 PCR was carried out on DNA extracted from MCMV-infected MEFs as described previously (Scott *et al.*, 2002). The entire M54 gene was amplified in four

Table 1. Antiviral susceptibilities of wild-type MCMV parent strain (Smith) and corresponding MCMV antiviral-resistant mutants

Antiviral	Mutant selection* (μM)	Parent IC_{50}† (μM)	Mutant IC_{50}† (μM)	Increase‡
ACV	60	1·2±0·1	26·0±2·5	21·7
ADV	60	0·5±0·1	135·1±70·1	270·2
CDV	8	0·2±0·2	7·0±3·9	35
GCV	80	6·5±2·0	51·8±18·4	6·7
PCV	160	8·1±1·7	22·2±4·8	2·7
PFA	800	80·9±14·7	439·8±65·1	5·4
VCV	240	2·3±0·6	279·1±140·2	121·3

*Final antiviral concentration for isolation of the antiviral resistant mutant.
†IC_{50}, 50 % inhibitory concentration (μM) of antiviral.
‡Indicates fold increase of mutant IC_{50} from that of the parent strain (Smith).

overlapping segments (M54A, M54B, M54C and M54D) of approximately 800 to 900 bp. M54A was amplified using primers M54.T8 (5′-ATCAATCGAGATAAGGGAGGG-3′) and M54.B7 (5′-TGGAGCCCTCGCCGCGAACGT-3′), M54B using primers M54.T6 (5′-CATAAGGGAAACGAACTACTT-3′) and M54.B5 (5′-CGACGGACAGCAGATCAGGAT-3′), M54C using primers M54.T4 (5′-GTTGGGCAAGATCATGTCCCG-3′) and M54.B3 (5′-AGAGCAGAGCAACTGGCCGTT-3′), and M54D using primers M54.T2 (5′-AAGGACAGGCAACGGTAGAAC-3′) and M54.B1 (5′-CTCCGATTTCGAGTACTGACG-3′). The entire M97 gene was amplified by PCR in three overlapping segments (M97A, M97B and M97C) of approximately 800 to 900 bp, using primers M97.T1 (5′-TCGGATCACTCTGGTGTGTG-3′) and M97.B7 (5′-AGCCCGCCGCTAGAGGAAGC-3′) for M97A, M97.T3 (5′-ATTCCGTGTCGGTCGCCCGGT-3′) and M97.B4 (5′-GGCCAGGCCCGCGTAGTCCC-3′) for M97B, and M97.T5 (5′-AGCGTCTACTGCAACATCCT-3′) and M97.B1 (5′-GACCGTCGTCGCATATCTGG-3′) for M97C. Each reaction consisted of 50 mM KCl, 10 mM Tris/HCl pH 9·0, 0·1 % Triton-X-100, 5 mM $MgCl_2$, 0·25 mM dNTP, 0·4 μM forward and reverse primer, 1 U *Taq* DNA polymerase (Promega) and 2 μl DNA template in a total reaction volume of 50 μl. Reaction conditions consisted of an initial denaturation step at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min.

M54 and M97 sequencing. PCR products were purified as described previously using polyethylene glycol (PEG) 8000 (Scott *et al.*, 2002), and sequenced using ABI Prism BigDye terminator chemistry (Applied Biosystems). Forward and reverse PCR primers were used for sequencing, as well as internal forward and reverse primers specific for each PCR product. The entire M54 and M97 gene sequences were assembled by comparison to Smith consensus sequences (Rawlinson *et al.*, 1996), translated using Alltrans and whole protein sequences for each gene aligned using CLUSTALW (Thompson *et al.*, 1994). The HCMV UL54 DNA polymerase and UL97 protein kinase sequences from laboratory strain AD169 (Chee *et al.*, 1990) and homologues of other herpesviruses were also included in the alignments for comparison. The DNA polymerase sequences of the nine wild-type MCMV strains and seven MCMV antiviral-resistant mutants were submitted to GenBank and have the accession numbers AY529137–AY529146 and AY529127–AY529133, respectively. The pM97 sequences from GCVres, PCVres and VCVres were also submitted to GenBank and have the accession numbers AY529134–AY529136.

DNA polymerase modelling. Protein structures with similar sequence to HCMV and MCMV DNA polymerase were identified by a BLAST search of the protein database (www.rscb.org/pdb) and these homologous polymerase proteins were aligned using CLUSTALX (Thompson *et al.*, 1997). This alignment included the bacteriophage RB69 DNA polymerase that has previously been used as a model for the analysis of herpes simplex virus and CMV DNA polymerase resistance mutations (Huang *et al.*, 1999; Chou *et al.*, 2003). An MCMV DNA polymerase model was built using the structure of the closest matched template of known three-dimensional structure, the Tgo polymerase from *Thermococcus gorgonarius* (PDB code 1TGO) (Hopfner *et al.*, 1999) with the help of COMPOSER as contained in Sybyl6.92 (Tripos). The model was subjected to energy minimization to optimize the geometry and remove steric overlap in the structure and Verify3D (Luthy *et al.*, 1992) was used to evaluate the quality of the model.

Growth kinetic assays. Growth kinetic assays comparing the wild-type parent strain (Smith) to each of the antiviral-resistant mutants were carried out in triplicate. Virus was inoculated at an m.o.i. of 0·01 p.f.u. per cell onto MEFs with centrifugal enhancement and cultured for 4 days. Cell-free (media) and cell-associated virus (trypsinized cells) were harvested and stored at −80 °C. Cell-free and cell-associated virus titres were quantified by calculation of tissue culture infective dose (TCID) in 96-well plates containing confluent MEFs. The number of infected cells per plaque for each MCMV strain was also counted after 4 days of culture to determine focus expansion and plaque size measured following photography. These analyses were performed as a blind trial.

RESULTS

Antiviral susceptibilities of the MCMV antiviral-resistant mutants

All seven MCMV antiviral-resistant mutants (ACVres, ADVres, CDVres, GCVres, PCVres, PFAres and VCVres) were isolated in antiviral concentrations that exceeded the IC_{50} and IC_{90} values of MCMV-sensitive strains (Rawlinson *et al.*, 1997; Smee *et al.*, 1995). PRA confirmed the MCMV mutants had significant increases in IC_{50} and IC_{90} values compared with those of the parent (Smith) strain (Table 1). The increases in antiviral inhibitory concentrations ranged from 2·7-fold for the PCVres mutant to 270·2-fold for the ADVres mutant compared with the parent (Smith) strain (Table 1).

Mutants generated against nucleoside analogues that require phosphorylation (ACV, GCV, PCV and VCV) also demonstrated cross-resistance to other antivirals within this group

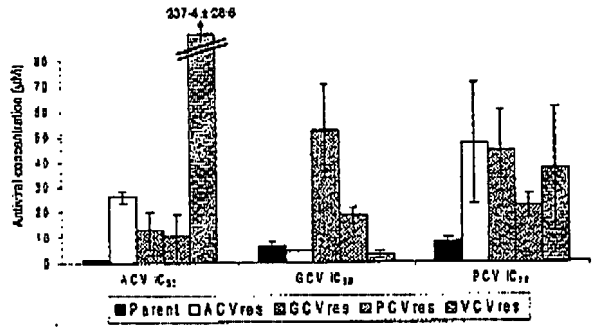

Fig. 1. MCMV mutants resistant to ACV (ACVres), GCV (GCVres), PCV (PCVres) and VCV (VCVres) are cross-resistant to other nucleoside analogue antivirals. The IC_{50} of ACV, GCV and PCV are shown with error bars for parent (Smith) strain and MCMV mutants resistant to ACV (ACVres), GCV (GCVres), PCV (PCVres) and VCV (VCVres). Large standard deviations were observed for PCV IC_{50} compared with other antivirals.

(Fig. 1). The GCVres and PCVres mutants demonstrated moderate levels of cross-resistance to ACV, with approximately 10-fold increases in IC_{50} values compared with parent (Smith) strain (Fig. 1), and 12- and 15-fold increases in IC_{90} values, respectively (results not shown). As expected, resistance to ACV was also observed for the MCMV mutant generated against VCV (Fig. 1). The level of resistance that VCVres demonstrated to ACV was very high, requiring inhibitory concentrations of ACV that were 200-fold that of the parent (Smith) strain, and 10-fold that of the ACVres mutant (Fig. 1). Cross-resistance to GCV was only observed with the PCVres mutant, and not ACVres or VCVres. All mutants tested (ACVres, GCVres and VCVres) demonstrated cross-resistance to PCV (Fig. 1).

MCMV DNA polymerase mutations associated with resistant phenotype

All seven antiviral-resistant mutants contained mutations of MCMV DNA polymerase (Table 2 and Fig. 2). These mutations occurred in regions of MCMV DNA polymerase that were invariant in nine sequenced wild-type (antiviral sensitive) strains (AY529137–AY529146) and mutations were verified by repeat amplification and sequencing reactions using a second set of different primers. No MCMV DNA polymerase mutations were found outside of codons 360–870 corresponding to the DNA polymerase domain region (Fig. 2), identified from alignment with HCMV sequences (Chou *et al.*, 1999). Half of the MCMV DNA polymerase mutations (4/8) occurred within or in close proximity to DNA polymerase domain III (Table 2 and Fig. 2).

The ACVres, ADVres and VCVres mutants contained identical amino acid mutations (P826R) between DNA polymerase functional domains I and VII (Table 2 and

Table 2. MCMV M54 (DNA polymerase) and M97 (protein kinase) mutations associated with antiviral resistance correlate with HCMV resistance mutations

MCMV mutant	Cross-resistance	DNA polymerase resistance mutations			Protein kinase resistance mutations		
		M54 (DNApol) mutation	DNA polymerase domain	HCMV correlates (resistance)*	M97 (PK) mutation	Protein kinase domain	HCMV correlates (resistance)*
ACVres	PCV	P826R	Between I and VII	V955I (VCV)[1]†	Nil	–	–
ADVres	ND	P826R	Between I and VII	V955I (VCV)[1]†	ND	–	–
CDVres	ND	L467I	3-region C	L501I (CDV, GCV)[5]	ND	–	–
		D709N‡	III	K805Q (CDV)[3]			
		V717L	III	V812L (GCV, CDV, PFA, ADV)[5]			
GCVres	ACV, PCV	L678V	VI	V781I (PFA, GCV)[1-4]	P479L	Between VIII and IX	–
		P754S	III	A834P (GCV, CDV, PFA)[1,5]			
PCVres	ACV, GCV	T753A‡	III	–	M395V	VIb	M460V (GCV)[5]
PFAres	301	A679V	VI	V781I (PFA, GCV)[1-4]	Nil	–	–
VCVres	ACV, PCV	P826R	Between I and VII	V955I (VCV)[1]†	T393M	VIb	M460V (GCV)[5]

*HCMV resistance mutations homologous or occurring in similar regions to the MCMV mutations ([1] Scott *et al.*, 2004; [2] Chiblar *et al.*, 1998a; [3] Chiblar *et al.*, 1998b; [4] Jabs *et al.*, 2001; [5] Chou *et al.*, 1995). MCMV resistance mutations with identical HCMV mutations are indicated in bold.

†These HCMV mutations have not been verified by marker transfer experiments.

‡MCMV mutations D709N and T753A reside 1 aa from the N terminus and 3 aa from the C terminus of DNA polymerase domain III, respectively.

ND, Not done.

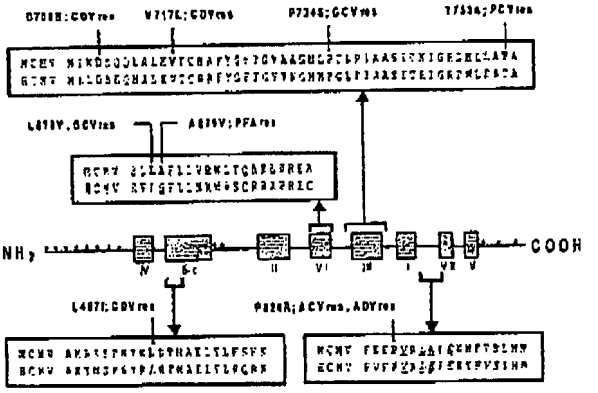

Fig. 2. DNA polymerase mutations associated with antiviral resistance. Fragments of the MCMV Smith sequence (Rawlinson *et al.*, 1996) and HCMV AD169 sequence (Chee *et al.*, 1990) are shown as the reference consensus sequences for selected DNA polymerase domains. Mutations associated with MCMV antiviral resistance are shown in bold, in relation to the domain or region in which they occur and their relationship with HCMV resistance mutations is shown in italics. A mutation found in a temperature-sensitive mutant (Ihara *et al.*, 1994) is shown underlined, and the IxIExE metal binding motif between DNA polymerase domains I and VII (Hopfner *et al.*, 1999) is shown in underlined italics. Regions of variation observed in antiviral-sensitive (wild-type) strains are indicated by an asterisk (*). Variation between domain VII and the C terminus occurred in only 5/11 of isolates.

Fig. 2). These mutants were isolated at different time points, and the ADVres mutation verified by repeat plaque purification of the mutant from original stocks and repeat sequencing. The CDVres mutant contained one mutation corresponding to DNA polymerase δ-region C (L467I) and two additional mutations at DNA polymerase domain III (D709N and V717L) (Table 2 and Fig. 2). The CDVres D709N mutation was located one codon outside the N-terminal end of DNA polymerase domain III, whereas mutation V717L lay in the centre of domain III. Two mutations were found in the GCVres mutant within DNA polymerase domains VI (L678V) and III (P734S) (Table 2 and Fig. 2). The only DNA polymerase mutation of PCVres (T753A) was located three codons outside of the C-terminal end of domain III (Fig. 2). A mutation within DNA polymerase domain VI was detected in the PFAres mutant (A679V), one residue downstream of the GCVres domain VI mutation (L678V) (Table 2 and Fig. 2).

Alignment of mutant MCMV and HCMV DNA polymerase sequences indicated two of the CDVres mutations (L467I and V717L) were identical in location and amino acid change to HCMV mutations (L501I and V812L) associated with antiviral resistance (Cihlar *et al.*, 1998a, b) (Table 2 and Fig. 2). The location of the other MCMV resistance mutations, except T753A of PCVres, also correlated with regions of HCMV DNA polymerase associated with antiviral resistance (Cihlar *et al.*, 1998a, b; Scott *et al.*, 2004). This included the third mutation of CDVres (D709N), located one codon upstream of the HCMV mutation K805Q associated with CDV resistance and the DNA polymerase domain III N terminus (Cihlar *et al.*, 1998a).

MCMV DNA polymerase three-dimensional structure

An MCMV DNA polymerase model generated using the *T. gorgonarius* Tgo polymerase crystal structure had good Verify3D scores for the majority of the protein (Fig. 3a). *T. gorgonarius* Tgo polymerase demonstrated greater similarity to HCMV and MCMV DNA polymerase than bacteriophage RB69 DNA polymerase, which has previously been used as a model for the study of herpes simplex virus and CMV antiviral resistance mutations (Huang *et al.*, 1999; Chou *et al.*, 2003). The catalytic domain regions of MCMV DNA polymerase exhibited the best Verify3D scores, and the N-terminal region of the protein model had the lowest score (Fig. 3a). A small number of insertions in the MCMV sequence relative to the template protein were not amenable to modelling and were removed from the model during alignment. These regions were predominantly within the N-terminal or extreme C-terminal region of MCMV DNA polymerase.

The MCMV DNA polymerase mutations associated with antiviral resistance in this study were analysed using the model (Fig. 3b). Only subtle perturbations in the MCMV DNA polymerase model were observed on alteration of the residues associated with antiviral resistance. However, most of the resistance mutations were located in the model surrounding an MCMV aspartate triad (D624, D791 and D793) homologous to the catalytic aspartate triad (D717, D910 and D912) of *T. gorgonarius* Tgo polymerase, a motif important for nucleotidyl transfer and metal binding (Rodriguez *et al.*, 2000).

M97 protein mutations associated with resistant phenotype

Mutants resistant to antivirals that require phosphorylation (PCVres, GCVres and VCVres) contained mutations of pM97 not present in MCMV antiviral-sensitive strains, in addition to the DNA polymerase mutations described above (Rawlinson *et al.*, 1997) (Table 2 and Fig. 4). Mutations of pM97 were not detected in the ACVres mutant, suggesting the P826R M54 (DNApol) mutation described above is solely responsible for the antiviral resistance of this isolate (Table 2 and Fig. 4). No pM97 mutations were detected in PFAres, demonstrating non-specific M97 mutations did not result from passaging of virus.

Regions of pM97 corresponding to protein kinase functional domains were defined by alignment with HCMV UL97 protein kinase, herpes simplex virus UL13 and other protein kinase sequences (Chee *et al.*, 1989; Hanks & Quinn,

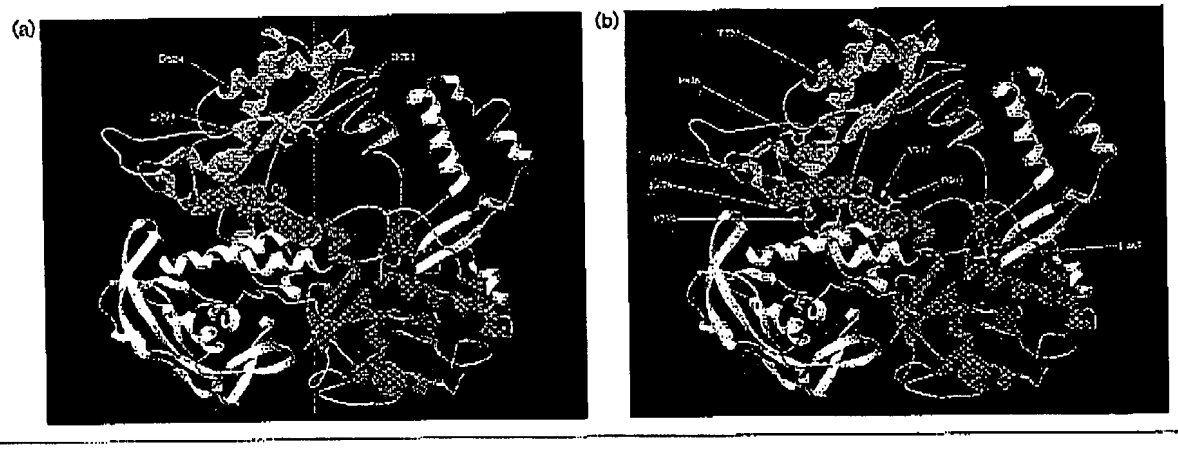

Fig. 3. MCMV DNA polymerase three-dimensional model. (a) A ribbon representation of MCMV DNA polymerase coloured according to region (yellow, N-terminal region; blue, exonuclease region; white, thumb region; green, palm region; red, finger regions) with the catalytic aspartate triad (D624, D791 and D793) drawn in bond representation coloured by atom type. (b) Ribbon model of MCMV DNA polymerase with residues identified as giving rise to drug resistance drawn in bond representation coloured by atom type.

1991). The pM97 mutations of the PCVres and VCVres mutants (M395V and T393M, respectively) occurred within regions corresponding to the protein kinase domain VIb conserved motif (*DxxxxN*) (Table 2 and Fig. 4). The M97 mutation of GCVres (P479L) was located between consensus motifs for protein kinase domains VIII (*APE*) and IX (*DxxxxG*) (Table 2 and Fig. 4). Alignment of the MCMV pM97 sequences with UL97 protein kinase sequences from HCMV antiviral-resistant isolates indicated the pM97 domain VIb mutation of PCVres was homologous to a mutation (M460V) that confers HCMV resistance to GCV, with the pM97 mutation of VCVres only two codons upstream of this site (Fig. 4) (Chou *et al.*, 1995).

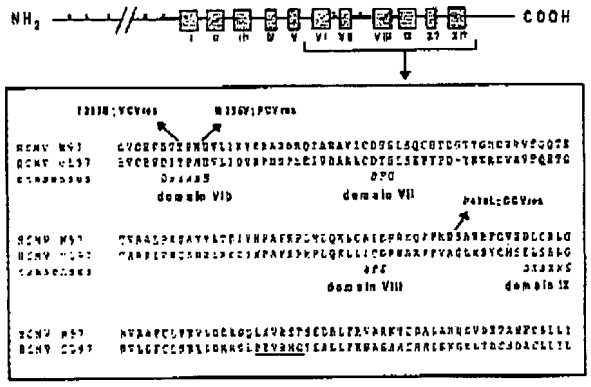

Fig. 4. MCMV pM97 and HCMV UL97 protein kinase antiviral resistance mutations. Alignment of the protein sequences of MCMV Smith M97 (Rawlinson *et al.*, 1996) and HCMV AD169 UL97 (Chee *et al.*, 1990) with MCMV resistance mutations identified in this study are indicated by bold font, and HCMV GCV resistance mutations from previous studies are indicated in italics (Chou, 1999; Erice, 1999). The putative domain IX consensus motif described by Chee *et al.* (1989) is underlined, although alignment with MCMV M97 suggests domain IX of UL97 is further towards the N terminus as indicated by consensus motif *DxxxxG* as position 488–493 of MCMV and 552–557 of HCMV. Regions of variation in wild-type antiviral-sensitive strains are indicated by an asterisk (*) (Rawlinson *et al.*, 1997).

Replication impairment of the MCMV antiviral-resistant mutants

All MCMV antiviral-resistant mutants were impaired in replication ability, producing smaller plaque sizes and 3- to 363-fold less cell-associated and cell-free virus titres than the parent (Smith) strain (Fig. 5). The most reduction in replication ability was observed with the VCVres mutant that contained single DNA polymerase and pM97 mutations (Fig. 5). The replication impairment of VCVres was greater than that observed for the ACVres and ADVres mutants (all containing identical DNA polymerase mutations), indicating the additional pM97 mutation of VCVres contributed to the overall decrease in replication ability of this isolate (Fig. 5 and Table 2). More than one mutation in MCMV DNA polymerase (the CDVres and GCVres mutants) or mutations in both DNA polymerase and pM97 (the GCVres, PCVres and VCVres mutants) tended to have a greater impact on replication ability than single DNA polymerase mutations. The PFAres mutant that contained a single DNA polymerase mutation corresponding to domain VI was the least replication-impaired mutant (three- to fivefold reduction in $TCID_{50}$) (Fig. 5). Only a moderate reduction in plaque size was observed for the PFAres mutant at day 4, and

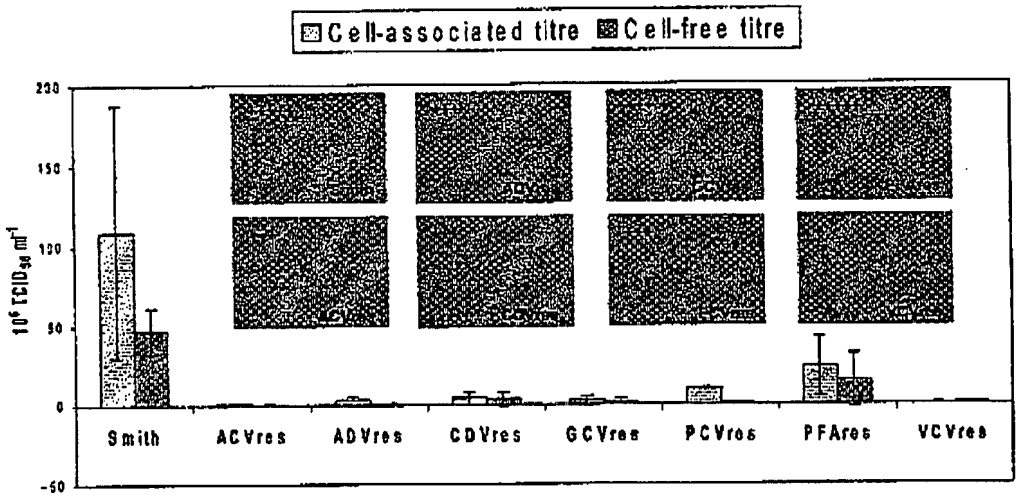

Fig. 5. Growth kinetics of MCMV parent (Smith) and antiviral resistant mutant strains in culture. The $TCID_{50}$ of cell-associated and cell-free MCMV parent (Smith) strain and antiviral resistant mutants after 4 days of culture in MEFs (initially inoculated at an m.o.i. of 0·01) are graphed. Insets are representative plaques produced by the parent (Smith) and antiviral resistant mutants after 7 days in culture with plaque size ratings (+, smallest; +++++, largest).

by day 7 the PFAres plaque sizes were equivalent to those observed for parent Smith strain (Fig. 5).

DISCUSSION

The antiviral-resistant and replication-impaired phenotypes of the seven MCMV mutants were associated with M54 DNA polymerase and the M97 putative protein kinase mutations that developed *in vitro* under selective pressure from antivirals. The involvement of the MCMV mutations in reduced antiviral susceptibility and replication ability is further supported by their correlation with HCMV mutations associated with antiviral resistance and their detection within protein regions of functional importance. In turn, the MCMV mutations associated with antiviral resistance and replication impairment have suggested important regions of MCMV DNA polymerase and pM97 that indicate functional elements of the HCMV and cellular homologues of these proteins.

The MCMV DNA polymerase mutation P826R between domains I and VII of the ACVres, ADVres and VCVres mutants was identical in position but different in substitution to a mutation (P826A) found in an ACV-resistant MCMV mutant generated in a separate study (Minematsu *et al.*, 2001). Furthermore, this MCMV mutation correlates with an HCMV DNA polymerase mutation (V955I) we have recently identified in a patient who developed an HCMV-related illness despite receiving VCV prophylaxis (Scott *et al.*, 2004). This suggests the region between DNA polymerase domains I and VII is potentially associated with CMV ACV susceptibility, which is enhanced for MCMV compared with HCMV (Cole & Balfour, 1987; Rawlinson *et al.*, 1997; Smee *et al.*, 1995). Domain I is the most conserved functional domain of DNA polymerases and is of major importance in substrate binding, polymerase activity and virus replication (Huang *et al.*, 1999; Ye & Huang, 1993). Domain VII is involved in polymerase activity and recognition of ACV (Hwang *et al.*, 1992; Ye & Huang, 1993). These two important domains are maintained as β-sheets in a herpesvirus DNA polymerase model (Huang *et al.*, 1999), and β-sheet turns are predominantly associated with proline (P) residues (Stryer, 1988). This suggests the antiviral resistance and replication impairment observed for ACVres, ADVres and VCVres might have resulted from altered interaction of DNA polymerase domains I and VII with nucleoside analogues and natural substrate.

The similarity observed between MCMV and HCMV mutations associated with antiviral resistance suggests MCMV antiviral-resistance and replication-impairment is potentially conferred via similar mechanisms as HCMV. Two mutations of CDVres (the δ-region C mutation L467I and domain III mutation V717L) were identical in location and amino acid substitution to HCMV DNA polymerase mutations that confer resistance to CDV and GCV (Cihlar *et al.*, 1998a, b; Lurain *et al.*, 1992). The L467I mutation is identical in position to a δ-region C mutation of HCMV DNA polymerase (L501F) that produces enhanced exonuclease activity (Kariya *et al.*, 2000), and presumably more efficient removal of incorporated inhibitor. The third CDVres mutation (D709N) aligned adjacent to the N-terminal codon of HCMV DNA polymerase domain III (K805), which when substituted with glutamine (Q) confers resistance to CDV (Cihlar *et al.*, 1998a). The D709N mutation is also congruent with domain III mutations of

HCMV DNA polymerase that interfere with DNA template binding (Ye & Huang, 1993). The P734S mutation of the GCVres mutant is in close proximity to an HCMV DNA polymerase mutation we have detected in an HCMV strain isolated from a patient demonstrating clinical resistance to GCV, PFA and CDV (Scott *et al.*, 2004). The T753A mutation of the PCVres mutant did not correlate with antiviral-resistant mutations of HCMV, but was in close proximity to ACV-resistant mutations of herpes simplex virus and varicella-zoster virus that result in decreased substrate binding (Huang *et al.*, 1999; Schmit & Boivin, 1999; Visse *et al.*, 1999). The A679V mutation of DNA polymerase domain VI was the only genetic alteration identified in the PFAres mutant, and corresponds in location with mutations of HCMV and other herpesvirus DNA polymerases associated with PFA-resistance (Cihlar *et al.*, 1998a; Schmit & Boivin, 1999). DNA polymerase domains II and VI are the only two functional domains of HCMV DNA polymerase associated with PFA-resistance (Cihlar *et al.*, 1998a). The GCVres mutant also contained a mutation (L678V) within MCMV DNA polymerase domain VI, and an association between this domain and HCMV GCV resistance has been suggested but not proven (Jabs *et al.*, 2001).

The conservative nature of almost all the resistance mutations identified in MCMV polymerase suggests that the mutations alter antiviral susceptibility of the polymerase via subtle perturbations of the protein. This is supported by analysis of the MCMV antiviral resistance mutations using the MCMV DNA polymerase model generated using the backbone of *T. gorgonarius* Tgo polymerase crystal structure (Hopfner *et al.*, 1999). Most of the resistance mutations in MCMV CDVres, GCVres, PCVres and PFAres are clustered in domains III and VI and surround a catalytic triad formed by conserved aspartic acid residues D624 (domain II), D791 and D793 (both in domain I). Analyses of *Thermococcus* sp. polymerases have demonstrated the importance of this catalytic aspartate triad for nucleotidyl transfer and Mg^{2+} binding (Rodriguez *et al.*, 2000). Therefore, the MCMV mutations potentially alter the local structure and hence orientation of the three aspartates in the triad, reducing the polymerase efficiency. This resulting reduction in polymerase efficiency would give the exonuclease domain an improved chance to remove any defective DNA strands containing antiviral nucleoside analogues, allowing the viral polymerase to complete replication successfully. A further potential mechanism of resistance involving disruption of an Mn^{2+}- and Zn^{2+}-binding site known to associate with the catalytic aspartate triad (Hopfner *et al.*, 1999) is also suggested by the P826R mutation present in MCMV ACVres, ADVres and VCVres given this residue's proximity to this metal-binding region. The position of L467I at the end of a helix in the nuclease domain suggests that this mutation may alter the processivity of the nuclease activity and potentially have a concomitant effect on the incorporation of inhibitors. Detailed study of the enzymology of the MCMV DNA polymerase will shed further light on the mechanism of action of these antiviral resistance mutations.

Interestingly, the two MCMV mutants generated against ACV (one against the ACV pro-drug VCV) produced identical DNA polymerase mutations (P826R), but the VCVres mutant also contained an additional mutation in M97. It is not known whether MEFs contain the specific dipeptide transporters responsible for the increased bioavailability of VCV in intestinal cells (Han *et al.*, 1998; Landowski *et al.*, 2003), nor VCV hydrolase required for cleavage of L-valine from VCV to produce ACV (Burnette *et al.*, 1995). It is therefore unclear whether these mechanisms contributed to the additional M97 mutation observed in the VCVres mutant, and no evidence exists suggesting the frequency of additional resistance mutations is related to exposure to higher antiviral concentrations. However, similar to results observed here with MCMV VCVres, HCMV isolates containing mutations in both DNA polymerase and UL97 have increased resistance to GCV compared with isolates containing single UL97 mutations (Smith *et al.*, 1997). In HCMV isolates, high-level resistance related to the length of exposure to antiviral (Smith *et al.*, 1997), whereas ACVres and VCVres were exposed to antiviral for similar lengths of time. The differences observed here for ACVres and VCVres were therefore most likely the result of random selection, enhanced by the process of plaque purification for each mutant.

The detection of three separate pM97 mutations in MCMV mutants resistant to ACV, GCV and PCV suggest the involvement of the putative M97-protein kinase in MCMV susceptibility to nucleoside analogues that require phosphorylation by virally encoded enzymes. Two of these mutations (T393M and M395V of the MCMV VCVres and PCVres mutants, respectively) reside within a region of pM97 homologous to the protein kinase domain VIb consensus motif (*DxxxxN*) important for phosphotransfer and catalytic activity (Chee *et al.*, 1989; Hanks & Hunter, 1995). The M395V mutation of the PCVres mutant is identical in position and amino acid substitution to the UL97 protein kinase mutation (M460V) frequently detected in GCV-resistant HCMV isolates (reviewed by Chou, 1999; Erice, 1999). The MCMV GCVres mutation (P479L) did not correlate with HCMV UL97 protein kinase mutations associated with antiviral resistance, and cannot be directly attributed to the observed phenotypic changes of this mutant due to the presence of two additional DNA polymerase mutations. However, the GCVres P479L mutation is located midway between protein kinase domain VIII (*APE*) involved in peptide recognition, and the peptide substrate binding domain IX (*DxxxxG*), and is therefore positionally central to the protein kinase catalytic core (Hanks & Hunter, 1995). No mutations were detected in the pM97 sequence (KTCDAL) homologous with the UL97 protein kinase AACRAL motif that has a strong association with antiviral resistance in HCMV GCV-resistant strains (Chou, 1999; Erice, 1999; Sullivan *et al.*, 1993). Furthermore, the domain VIb mutation of MCMV VCVres did not confer cross-resistance to GCV despite being homologous to an HCMV UL97 protein kinase mutation that is common in

HCMV GCV-resistant isolates (Chou, 1999; Erice, 1999). This difference may account for the low levels of GCV phosphorylation by MCMV pM97 compared with HCMV UL97 (Wagner *et al.*, 2000). However, the low levels of phosphorylated GCV produced in MCMV-infected cells are sufficient for inhibition of MCMV replication, as shown in this study and others (Rawlinson *et al.*, 1997; Smee *et al.*, 1995; Wagner *et al.*, 2000), suggesting MCMV DNA polymerase has a high affinity for trace levels of phosphorylated nucleoside analogues produced by the M97 putative protein kinase (Wagner *et al.*, 2000).

MCMV and HCMV are similar in the DNA polymerase and protein kinase mutations that develop under antiviral selective pressure, the evidence suggesting potential involvement of the respective protein kinase homologues in antiviral susceptibility to nucleoside analogues, and the DNA polymerase and protein kinase regions that appear to be of functional importance. These similarities indicate a close relationship between MCMV and HCMV antiviral resistance and demonstrate the utility of MCMV as an animal model for CMV antiviral studies.

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Evaluations of Unformulated and Formulated Dendrimer-Based Microbicide Candidates in Mouse and Guinea Pig Models of Genital Herpes

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Prevention of sexually transmitted infections is a priority in developed and developing countries. One approach to prevention is the use of topical microbicides, and one promising approach is the use of dendrimers, highly branched macromolecules synthesized from a polyfunctional core. Three new dendrimer products developed to provide stable and cost-efficient microbicides were initially evaluated in vitro for anti-herpes simplex virus activity and then in vivo by using a mouse model of genital herpes. From these experiments one product, SPL7013, was chosen for further evaluation to define the dose and duration of protection. Unformulated SPL7013 provided significant protection from genital herpes disease and infection at concentrations as low as 1 mg/ml and for at least 1 h following topical (intravaginal) administration of 10 mg/ml. This compound was then formulated into three vehicles and further evaluated in mouse and guinea pig models of genital herpes infection. In the murine evaluations each of the formulations provided significant protection at concentrations of 10 and 50 mg/ml. Formulated compounds provided protection for at least 1 h at a concentration of 10 mg/ml. From these experiments formulation 2V was chosen for dose ranging experiments using the guinea pig model of genital herpes. The guinea pig evaluations suggested that doses of 30 to 50 mg/ml were required for optimal protection. From these studies a lead compound and formulation (2V of SPL7013) was chosen for ongoing evaluations in primate models of simian immunodeficiency virus and *Chlamydia trachomatis* infection.

The spread of sexually transmitted infections (STIs) continues to grow at an alarming rate. In the United States more than 12 million people are infected with STIs every year, accounting for 5 of the 10 most commonly reported infectious diseases (4). Globally the incidence of human immunodeficiency virus (HIV) infection continues to grow, with the most recent data from the United Nations showing that 40 million people worldwide are HIV positive. Similarly, infections with herpes simplex virus type 2 (HSV-2) continue to increase around the world at an alarming rate despite the availability of effective antivirals (11). Seroprevalence data suggest that >45 million patients are infected in the United States at this time, with projections for even further increases (5, 6). The high percentage of women infected with both HSV-2 and HIV is of particular concern. Because genital herpes can lead to an increased risk of HIV infections, prevention of genital herpesvirus infections may also impact the spread of HIV (5). Vaccines for STIs remain an important goal for reduction of the their spread; however, HIV vaccines remain an elusive goal, while the prospects for vaccines for other STIs, including genital herpesvirus and human papillomavirus infection, are more encouraging (8, 12).

Microbicides, defined as a chemical entity that can prevent or reduce transmission of STIs when applied to the vagina or rectum, represent an intriguing approach to the prevention of STIs. Most microbicide candidates act by disrupting the cell membrane or envelope of the pathogen (for example, detergents such as nonoxynol-9), by blocking receptor-ligand interactions (for example, sulfated compounds, such as PRO 2000), or by modifying the vaginal environment (for example, pH buffering agents such as Buffer-gel) (reviewed in references 10, 14, and 15).

Dendrimers are a relatively new class of macromolecules characterized by highly branched three-dimensional architectures that offer an alternative to polyanionic polymers. They are assembled in a precise stepwise manner, and this controlled synthesis allows the assembly of highly defined "nanoobjects," in contrast to the heterogenous nature of traditional polymer-based materials. Therefore, we applied this technology to prepare defined macromolecular polyanions that would retain good levels of activity against the early stages of viral infection and have optimum physical properties (i.e., low systemic absorption, water solubility, ease of formulation, etc.) for microbicide development. In vitro and in vivo studies on a selection of these compounds have been reported previously and showed that they are potent inhibitors of a range of sexually transmitted diseases. Several compounds inhibited the replication of HIV type 1 with a 50% effective concentration (EC_{50}) of <1 μg/ml (19), while members of this same class of dendrimer were also effective in vitro against HSV-1 and HSV-2 (3). These compounds appeared to inhibit the early stages of virus replication although there was some evidence of effects on the late stages of viral replication (17, 19). In addi-

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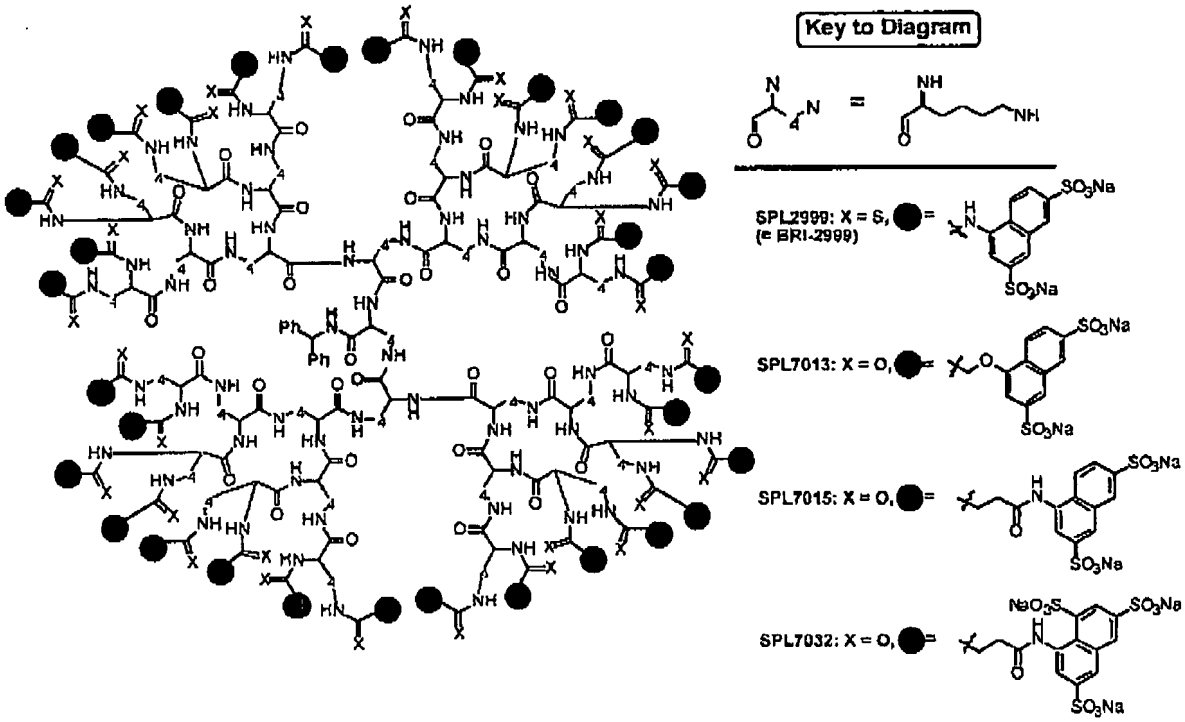

FIG. 1. Molecular structures of SPL2999, SPL7013, SPL7015, and SPL7032. The lysine dendrimer generations are built out from a central core (boldface), through a series of layers or generations. The outer sulfonic acid surface is attached via thiourea (SPL2992) or amide (SPL7013, SPL7015, and SPL7032) linkages.

tion, the compounds were nontoxic to the cells up to the highest concentration tested, 100 μg/ml (3).

Recently, dendrimers dissolved in saline (i.e., unformulated) were used in in vivo evaluations of activity in a mouse model of genital herpes (3). These early dendrimer-based microbicide candidates were prepared by assembling aromatic-sulfonic acid or aromatic-carboxylic acid units to the outer surface of lysine- or polyamidoamine-based dendrimers via a thiourea linkage (for example, see compound SPL2999 in Fig. 1, which has previously been referred to as BRI-2999) (3, 7). Since that time we have further refined the dendrimer architecture in order to produce compounds in a Good Laboratory Practice/Current Good Manufacturing Practice environment with the required stability and cost-of-production profile necessary for a microbicide candidate. This work resulted in the identification of three new microbicide candidates, SPL7013, SPL7015, and SPL7032 (Fig. 1), which are all prepared from the same lysine dendrimer and where the earlier thiourea linkage has been replaced by a more stable amide bond.

This paper reports on the in vivo evaluation of these stable, expanded-spectrum dendrimers when used in mouse and guinea pig models of HSV-2-induced genital herpesvirus infection. In addition we report the in vivo activity of three prototype formulations of the selected development candidate, SPL7013. This formulated preparation is currently undergoing in vivo evaluations in macaques for efficacy against simian/human immunodeficiency virus and *Chlamydia trachomatis*.

MATERIALS AND METHODS

Viruses. HSV-2 strain G was used for in vitro assays (7), while HSV-2 strain 186 was used for mouse inoculations (3) and strain MS was used for guinea pig inoculations (2). All viruses were prepared as described previously (2, 3, 7).

Dendrimers. A powder form of the dendrimers was supplied by Starpharma (Prahran, Australia). Solutions (1 to 100 mg/ml) were prepared in phosphate-buffered saline (PBS) and stored at room temperature.

Formulations. Formulation development work on SPL7013 was performed at the Center for Pharmaceutical Science and Technology at the University of Kentucky. Methylparaben (NF), propylparaben (NF), edetate disodium dihydrate (EDTA; USP), propylene glycol (USP), glycerin (USP), and sodium hydroxide (NF) were obtained from Spectrum Quality Products, Inc. (New Brunswick, N.J.). Carbopol 971P (NF) was purchased from BF Goodrich Specialty Chemicals (Cleveland, Ohio).

A range of excipients were initially investigated, but ultimately research focused on carbopol-based aqueous gels due to their mucoadhesive properties and their use in vaginal products and other microbicide formulations. As shown in Table 1, three different gel prototype SPL7013 formulations (1V, 2V, and 3V) were developed containing 5, 1, 0.1, and 0% (wt/wt) SPL7013. The prototypes differed only in the final percentage (weight per weight) of propylene glycol and glycerin. All of the gel formulations contained a final carbopol 971P (NF) concentration of 4.75 to 5% (wt/wt) depending on the final concentration of SPL7013 in the gel. Carbopol 971P (NF) is a cross-linked acrylic acid listed in the *USP Monograph* as Carbomer 941. After initial evaluations, 1 to 5% SPL7013-containing formulation 2V was also prepared.

The gels were made with a Caframo (Wiarton, Ontario, Canada) stirrer, model BDC-1850, by adding the required amount of purified water, EDTA, and then propylene glycol and glycerin. Next, carbopol 971P (NF) was slowly added to avoid clumping, and the formed gel was mixed until the polymer was fully hydrated (~1 h). The pH was adjusted to 4.5 with 2 N NaOH, and then methylparaben and propylparaben were added and mixed until dissolved. The gel was then made to weight with purified water, and the final pH was adjusted to 4.5

TABLE 1. Excipients for three prototype vaginal microbicide placebo gels

Excipient	Prototype gel	Amt (wt/wt) (%)	Function
Water for injection, USP	All	100[a]	Vehicle
Methylparaben, NF	All	0.18	Antimicrobial preservative
Propylparaben, NF	All	0.02	Antimicrobial preservative
EDTA, USP	All	0.1	Antioxidant
Carbopol 971, NF	All	5.0	Gelling agent
Propylene glycol, USP	1V	5.0	Emollient
	2V	1.0	
	3V	0	
Glycerin, USP	1V	5.0	Emollient
	2V	1.0	
	3V	0	
2 N NaOH to pH 4.5	All	9.0	pH-adjusting agent

[a] Make up to 100%.

with either 2 N NaOH or 1 N HCl. The viscosity of all gels was measured with a cone and plate rheometer, model RVDV III+ (Brookfield Engineering; Middleboro, Mass.), at 25°C for 5 min at 1.7 rpm by using spindle CPE-52. For all gels, the viscosity of the formulations was in the range of 30,000 to 43,000 cP under the conditions described above.

Each formulation was assessed for toxicity in a 5-day rabbit vaginal-irritation study prior to evaluation. Each of the placebo prototype gels and those gels containing 1 and 5% (wt/wt) SPL7013 elicited the same level of minimal irritation in a 5-day repeat dose rabbit vaginal model (data not shown).

In vitro evaluations. Confluent Vero cell monolayers in six-well plates were incubated in duplicate with different concentrations of dendrimers ranging from 0.01 to 30 μg/ml at 37°C for 1 h. One hundred PFU of HSV-2 strain G were then added to the cells, and the samples were incubated at 37°C for 1 h. After the inoculum was removed, the cells were washed with PBS and overlaid with 0.5% methylcellulose for a plaque assay. After 2 days the monolayers were fixed with 10% formalin and stained with 0.5% crystal violet as previously described (7). EC_{50} values were calculated with the Statview computer program.

The cytotoxicity of the compounds was also evaluated by using Vero cells following incubation with various concentrations of the test compounds for 2 days and examination using the neutral-red uptake assay as previously described (7).

Animal models. All animal protocols were approved by the Cincinnati Children's Hospital Animal Use and Care Committee. All procedures complied with the relevant federal and institutional policies.

Mouse model of genital HSV-2 infection. As previously described (2, 3) female Swiss Webster mice weighing 18 to 21 g (Harlan, Indianapolis, Ind.) were given 0.1 ml of a suspension containing 3 mg of medroxyprogesterone acetate (Upjohn Pharmacia, Kalamazoo, Mich.) by subcutaneous injection 7 days and 1 day prior to challenge to increase susceptibility to vaginal HSV infection. Animals were then anesthetized, and the vaginas were swabbed with a calcium alginate swab prior to intravaginal inoculation of the formulated or unformulated dendrimer or placebo in a volume of 15 μl. Following various defined intervals the animals were then challenged with 15 μl of a suspension containing 10^4 PFU of HSV-2 strain 186 applied intravaginally without removal of the preceding treatment material. Vaginal swabs were collected from all animals on day 2 after inoculation and stored frozen (−80°C) until assayed for the presence of virus on susceptible rabbit kidney cells. Animals were then monitored daily for 21 days for evidence of herpetic disease, including hair loss and erythema around the perineum, chronic urinary incontinence, hind-limb paralysis, and death. For the purpose of these studies animals that did not develop symptoms were defined as infected if virus was isolated from the vaginal swab specimens collected on day 2 after inoculation (2, 3).

Guinea pig model of genital herpes. As previously described, Hartley guinea pigs weighing 275 to 300 g (Charles River Breeding Laboratory, Wilmington, Mass.) were treated intravaginally with 200 μl of formulated dendrimer or placebo, followed by intravaginal inoculation with 200 μl of a suspension containing 10^6 PFU of HSV-2 strain MS without removal of the preceding treatment material (2). Vaginal swabs were obtained on days 1 and 2 postinoculation and stored frozen (−80°) until assayed for the presence of virus on susceptible rabbit kidney cells. For the purpose of these studies animals that did not develop symptoms were defined as infected if virus was isolated from the vaginal swab specimens collected on day 1 or 2 after inoculation (2).

Statistics. Incidence data were compared by Fisher's exact test. All comparisons were two-sided. No corrections were made for multiple comparisons.

TABLE 2. Antiviral activity of dendrimers against HSV-2 determined by plaque reduction assay[a]

Dendrimer	EC_{50} (μg/ml)	CC_{50} (μg/ml)[b]
SPL7013	0.6[c]	>1,000
SPL7015	0.5	>1,000
SPL7032	0.7	>1,000

[a] Evaluations were repeated five times for SPL7013, twice for SPL7015, and once for SPL7032.
[b] CC_{50}, cytotoxic concentration.
[c] The standard deviation was 0.03 μg/ml.

TABLE 3. Evaluation of three expanded-spectrum dendrimer products against genital herpes in mice[b]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013	100	11/11 (100)[a]	11/11 (100)[a]
SPL7015	100	9/12 (75)[a]	8/12 (67)[a]
SPL7032	100	11/12 (92)[a]	11/12 (92)[a]
PBS		0/12 (0)	0/12(0)

[a] $P < 0.005$ versus PBS.
[b] Mice were treated 20 s prior to challenge.

RESULTS

In vitro. All three compounds had similar in vitro activities against HSV-2, with no evidence of toxicity even at the highest concentration tested, 1,000 μg/ml (Table 2).

Animal models. (i) Unformulated dendrimers. In the initial experiment 10% solutions of SPL7013, -7015, and -7032 (Fig. 1) were evaluated in mice. Significant protection by each compound against disease and infection was observed (Table 3) when the time from treatment to virus challenge was minimal (20 s). From this and similar comparisons and because of the ease of manufacturing, cost, and stability, SPL7013 was chosen for further development.

In the subsequent experiments either the effect of drug concentration or the duration of protection was evaluated. As seen in Table 4 compound SPL7013 provided significant protection at concentrations as low as 1 mg/ml when the time from treat-

TABLE 4. Effect of concentration on protection from genital herpes by dendrimer SPL7013 in mice[c]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013	100	12/12 (100)[a]	12/12 (100)[a]
SPL7013	10	11/12 (92)[a]	10/12 (83)[a]
SPL7013	1	8/12 (67)[b]	6/12 (50)[a]
PBS		0/12 (0)	0/12(0)

[a] $P < 0.001$ versus PBS.
[b] $P < 0.05$ versus PBS.
[c] Mice were treated 20 s prior to challenge.

TABLE 5. Duration of protection by dendrimer SPL7013 against genital herpes in mice

Treatment	Concn (mg/ml)	Time (min) treated prior to challenge	Fraction (%) of animals protected against:	
			Disease	Infection
SPL7013	10	5	14/16 (88)[a]	14/16 (88)[a]
SPL7013	10	30	13/16 (81)[a]	12/16 (75)[a]
PBS		5	0/16 (0)	0/16 (0)
SPL7013	10	60	5/15 (33)[b]	4/15 (27)
PBS		5	0/15 (0)	0/15 (0)

[a] $P < 0.001$ versus PBS.
[b] $P < 0.05$ versus PBS.

ment to challenge was minimal. As seen in Table 5 this compound, at a concentration of 10 mg/ml, provided significant protection from disease for at least 1 h following administration.

(ii) Formulated dendrimers. Three different formulations of dendrimer SPL7013 were then prepared at the University of Kentucky at concentrations of 1 and 5% (Table 1). In the initial experiment each formulation of 1% SPL7013 was evaluated in the mouse model of genital HSV infection. As seen in Table 6 each formulation provided significant protection when administered 5 min prior to intravaginal challenge. Note also that the placebo formulation provided some protection against disease but not infection. This is most likely due to the buffering effect of the formulation in maintaining the acid pH of the vagina. In the subsequent experiment the duration of protection out to 30 min after treatment with the 5% concentration of each formulation was evaluated. Again, significant protection against infection and disease was provided by each formulation (Table 7). The 2V formulation was chosen for further evaluation and was shown to provide significant protection at a concentration of 1% for 30 min in two experiments and for at least 1 h after application in the one experiment where this was evaluated (Table 8).

The 2V formulation of SPL7013 was further evaluated in the guinea pig model of genital herpes because this model, it is felt, better mimics human disease (13). In the initial experiments 1 to 5% concentrations of SPL7013 in formulation 2V were applied 5 min prior to virus challenge. As seen in Table 9, protection appeared to be dose dependant, with increased protection at 3 to 5% concentrations. The experiment was repeated to determine if the decreased activity of the 30-mg/ml dose would be confirmed. Repeat experiments showed that protection with this concentration was not diminished in comparison to that with lesser concentrations. Thus, the second experiment confirmed the high protection rates provided by the 3 and 5% concentrations and were consistent with dose-dependant activity. The activity seen in the placebo recipients in the first experiment is consistent with that observed in some of the mouse studies (Table 6) with formulation 2V.

TABLE 6. Evaluation of three 1% formulations of dendrimer SPL7013 against genital herpes in mice[c]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013 formulation 1V	10	11/16 (69)[a,e]	11/16 (69)[a,d]
Placebo 1V		3/16 (19)	1/16 (6)
SPL7013 formulation 2V	10	12/16 (75)[a]	12/16 (75)[a,e]
Placebo 2V		7/16 (44)[b]	4/16 (25)
SPL7013 formulation 3V	10	13/15 (87)[a,e]	12/15 (80)[a,e]
Placebo 3V		6/16 (38)[b]	4/16 (25)
PBS		1/16 (6)	1/16 (6)

[a] $P < 0.001$ versus PBS.
[b] $P < 0.05$ versus PBS.
[c] $P < 0.05$ versus placebo.
[d] $P < 0.001$ versus placebo.
[e] Mice were treated 5 min prior to challenge.

TABLE 7. Evaluation of three 5% formulations of dendrimer SPL7013 against genital herpes in mice

Treatment	Concn (mg/ml)	Time (min) treated prior to challenge	Fraction (%) of animals protected against:	
			Disease	Infection
SPL7013 formulation 1V	50	30	10/16 (63)[a]	10/16 (63)[a]
SPL7013 formulation 2V	50	30	15/16 (94)[a]	15/16 (94)[a]
SPL7013 formulation 3V	50	30	16/16 (100)[a]	16/16 (100)[a]
SPL7013 formulation 1V	50	5	14/16 (88)[a]	13/16 (81)
SPL7013 formulation 2V	50	5	15/16 (94)[a]	15/16 (94)[a]
SPL7013 formulation 3V	50	5	15/16 (94)[a]	15/16 (94)[a]
PBS		5	0/16 (0)	0/16 (0)

[a] $P < 0.001$ versus PBS.

DISCUSSION

The continued HIV epidemic and ongoing increases in the prevalence of genital HSV-2 and other STIs underscore the need for a safe effective user-controlled strategy to prevent these infections. Microbicides offer one such strategy. Because of the lack of efficacy and possible deleterious effects of N-9, a nonionic surfactant that disrupts lipid membranes, such as viral envelopes (16, 18), compounds that inhibit binding, such as polyanions, rather than acting as detergents are receiving increased attention (reviewed in references 10, 14, and 15). One potential drawback of the polyanions in clinical development as topical microbicides is that they are mixtures of compounds. PRO 2000 (1, 9), for example, is a polymer mixture of between 4 and 6 kDa, and Carraguard contains various carbohydrates with various levels of sulfation. In contrast, SPL7013 has been characterized by mass spectrometry, capillary electrophoresis,

TABLE 8. Evaluation of duration of protection of 1% 2V formulation of SPL7013 against genital herpes in mice

Treatment	Concn (mg/ml)	Time (min) treated prior to challenge	Fraction (%) of animals protected against:	
			Disease	Infection
SPL7013 formulation 2V	10	5	8/15 (53)[a]	8/15 (53)[a]
SPL7013 formulation 2V	10	30	9/15 (60)[a]	8/15 (53)[a]
SPL7013 formulation 2V	10	60	6/15 (40)[b]	6/15 (40)[b]
PBS		5	0/15 (0)	0/15 (0)
SPL7013 formulation 2V	10	5	8/12 (67)[a]	8/12 (67)[a]
SPL7013 formulation 2V	10	30	8/12 (67)[a]	8/12 (67)[a]
PBS		5	0/12 (0)	0/12 (0)

[a] $P < 0.01$ versus PBS.
[b] $P < 0.05$ versus PBS.

TABLE 9. Evaluation of protection of different concentrations of formulation 2V of SPL7013 against genital herpes in guinea pigs[a]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013 formulation 2V	10	7/15 (47)	5/15 (33)
SPL7013 formulation 2V	20	10/15 (67)	9/15 (60)[c]
SPL7013 formulation 2V	30	7/15 (47)	7/15 (47)[c]
SPL7013 formulation 2V	40	11/15 (73)[c]	10/15 (67)[c]
SPL7013 formulation 2V	50	12/15 (80)[c]	11/15 (73)[c]
Placebo gel		10/15 (67)	8/15 (53)[c]
PBS		4/15 (27)	1/15 (7)
SPL7013 formulation 2V	30	16/18 (89)[b,c]	15/18 (83)[b,c]
SPL7013 formulation 2V	50	17/18 (94)[b,c]	16/18 (89)[b,c]
Placebo gel		3/18 (17)	2/18 (11)
PBS		4/18 (22)	3/18 (17)

[a] $P < 0.05$ versus PBS.
[b] $P < 0.001$ versus PBS.
[c] $P < 0.001$ versus placebo.
[d] Animals were treated 5 min prior to challenge.

and high-pressure liquid chromatography, and in-process controls have been developed to tightly control the synthesis. As a result SPL7013 has entered full preclinical development as a topical microbicide.

In this paper we have shown that dendrimer SPL7013 provides protection from infection and disease in the mouse model of genital herpes even at concentrations as low as 1 mg/ml and for at least 1 h after administration. Similarly, after formulation this candidate microbicide remained active when used in the guinea pig model of genital herpes. Thus, despite the increased size, vaginal vault area, and higher dose of virus used in the guinea pig model, the high activity was maintained. Note also that, although good activity was maintained after formulation, there was no obvious advantage to the formulated product. Continuing evaluations are aimed at determining if the formulated products have advantages either in the duration of protection or dose effects in both the mouse and guinea pig models. Further, whether there might be advantages in larger animals, such as the primates that are currently being evaluated and humans, remains to be determined. The goal of the formulation should be to increase the spread of the material so it is more effective, increase the time it is present in the vaginal cavity through mucoadhesive or other properties to increase the duration of protection, or provide additional activity, for example, by maintaining the vaginal pH.

From both the mouse and guinea pig evaluations it appears that concentrations of 3% or higher of the formulated product may be necessary for optimal protection. Because of the encouraging results with this formulated dendrimer in the experiments presented here, evaluations in monkey models of simian/human immunodeficiency virus and chlamydia are ongoing. Dendrimer SPL7013 is one of the leading candidates to fulfill the difficult requirements of a microbicide to be safe yet active against a number of STIs.

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CONCISE COMMUNICATIONS

The Topical Microbicide PRO 2000 Protects against Genital Herpes Infection in a Mouse Model

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Vaginal gel formulations containing the naphthalene sulfonate polymer PRO 2000 are being developed as topical microbicides to protect against infection with sexually transmitted disease (STD) pathogens. A mouse model was used to determine whether PRO 2000 could protect against genital herpes in vivo. Animals received a single intravaginal application of 15 μL of a 10% PRO 2000 aqueous solution or a 4.0% or 0.5% PRO 2000 vaginal gel formulation 20 s prior to intravaginal challenge with 4.0 $\log_{10}$ pfu of herpes simplex virus type 2. Treatment with the 4.0% gel provided complete protection against infection; treatment with the 0.5% gel or 10% solution provided 81% and 80% protection, respectively. Furthermore, the 4% gel provided significant protection even when viral challenge was delayed until 60 min after treatment. This is the first report to show that PRO 2000 can protect against infection with an STD pathogen in vivo.

The incidence of sexually transmitted diseases (STDs) continues to rise at an alarming rate. Currently 5 of the 10 most commonly reported infectious diseases in the United States are sexually transmitted, and an estimated 12 million cases of STDs occur annually [1]. Topical microbicides, which are applied directly to the genital tract on an episodic basis and protect against infection, are an attractive approach to reducing the spread of STDs. The fact that microbicide use can be female initiated (if necessary, without partner consent) has added impetus to the search for safe and effective compounds, because it is recognized that females bear a disproportionate burden of STD infection and are frequently unable to negotiate condom use [1–3]. To date there have been two main approaches to the development of topical microbicides. The first has been to identify detergents or surface active agents that inactivate STD pathogens by disruption of the outer envelope or membrane. The compound that has been most fully evaluated in this regard is the detergent nonoxynol-9 (N-9), which is the active ingredient in many over-the-counter spermicides [4–6].

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The second strategy has been to identify compounds that can prevent infection by blocking binding of the pathogen to host cells. The naphthalene sulfonate polymer PRO 2000 (Procept, Cambridge, MA) is an example of this second approach. PRO 2000 was originally found to disrupt early molecular events in the human immunodeficiency virus (HIV) type 1 infection process and to suppress infection by a broad range of HIV isolates [7]. More recent studies have shown that the compound is also active in vitro against two other common STD pathogens: herpes simplex virus type 2 (HSV-2; 50% effective concentration [EC_{50}] < 0.03 μg/mL) and *Chlamydia trachomatis* (EC_{50} 0.6 μg/mL) [8]. Thus, it has the potential to act as a broad-spectrum microbicide. In addition, PRO 2000 has a number of other characteristics that are desirable in a microbicide. It is straightforward to synthesize, highly water soluble and stable, virtually colorless and odorless, and compatible with latex condoms. Furthermore, two recent phase 1 clinical trials showed that vaginal gel formulations containing ≤4% of PRO 2000 are safe and well tolerated (unpublished data).

In the studies reported here, we examined the ability of PRO 2000 both in solution and in the vaginal gel formulations used in recent clinical trials to act as a topical microbicide against HSV-2 infection in vivo in a mouse model of genital herpes.

Materials and Methods

PRO 2000. PRO 2000 powder was synthesized by the polymerization of 2-naphthalene sulfonic acid and formaldehyde, followed by the selective precipitation of a 5 ± 1 kDa low-polydispersity fraction. The 4.0% and 0.5% (wt/wt) PRO 2000 vaginal gel formulations were prepared by combining PRO 2000 powder in water with 2.0% and 1.35% Carbopol 1382 (B. F. Goodrich, Cleve-

Table 1. Effect of PRO 2000 against genital herpes simplex virus type 2 in mice.

Series	PRO 2000 concentration (%)	Vehicle	No. inoculated	No. protected against infection[a] (%)	No. protected against disease (%)
1	0	PBS	15	0	1 (7)
	10	PBS	15	12 (80)[b]	12 (80)[b]
	0	Gel	15	2 (13)	2 (13)
	4	Gel	15	15 (100)[b]	15 (100)[b]
2	0	Gel	17	3 (18)	4 (24)
	0.5	Gel	16	13 (81)[b]	14 (88)[b]
	4	Gel	16	16 (100)[b]	16 (100)[b]

NOTE. All mice were treated for −20 s relative to virus inoculation.

[a] Animals that did not develop symptoms were defined as infected if virus was isolated from vaginal swabs collected on day 2 after inoculation.

[b] $P < .001$ vs. appropriate control by Fisher's exact test.

land, OH), respectively, 0.05% lactic acid, and trolamine (to pH 4.5). A placebo gel of comparable viscosity was prepared by use of 1.0% (wt/wt) Carbopol 1382. The powder and gel formulations were supplied by Procept.

Viruses and cells. HSV-2 strain 186 [9] was prepared by culture in low-passage primary rabbit kidney (RK) cells. Virus stocks were maintained frozen (−80°C). RK cells were prepared as previously described [10] and maintained in Eagle's basal medium supplemented with 10% (vol/vol) fetal bovine serum.

Mouse model of genital HSV-2 infection. Female Swiss Webster mice weighing 18–21 g (Harlan, Indianapolis) were administered 0.1 mL of a suspension containing 3 mg of medroxyprogesterone acetate (Upjohn Pharmacia, Kalamazoo, MI) by subcutaneous injection in the shoulder region 7 and 1 days prior to viral challenge, to increase susceptibility to vaginal HSV infection. On the day of viral challenge, animals were anesthetized by intraperitoneal injection of 0.25 mL of a solution containing 6.5 mg/mL sodium pentobarbital. The vaginal vault was swabbed twice, first with a moistened type 1 calcium alginate–tipped swab (Fisher Scientific, Pittsburgh) and then with a dry swab. Animals were then inoculated by instillation of 15 μL of a suspension containing 4.0 $\log_{10}$ pfu of HSV-2 186.

Vaginal swab samples were collected from all animals on day 2 after inoculation and stored frozen (−80°C) until assayed for the presence of virus by culture on susceptible RK cell monolayers. Mice were evaluated daily, to day 21 after inoculation, for evidence of symptomatic infection that included hair loss and erythema around the perineum, chronic urinary incontinence, hind-limb paralysis, and mortality. For the purpose of these studies, animals that did not develop symptoms were defined as infected if virus was isolated from vaginal swab samples collected on day 2 after inoculation.

Statistics. Incidence data were compared by Fisher's exact test. All comparisons were two-tailed.

Results

In the initial study (table 1; series 1), groups of mice were treated intravaginally with 15 μL of a 10% (wt/vol) PRO 2000 solution in sterile PBS or with 15 μL of 4% PRO 2000 gel 20 s prior to virus inoculation. Control animals received the same volume of sterile PBS or the placebo gel formulation. The incidence of infection was comparable in both control groups (15/15 PBS vs. 13/15 placebo: 28 of 30 animals were infected in total). Subsequently, all but 1 of these mice developed symptoms and died, demonstrating that the placebo gel provided no protection against viral challenge. In contrast, PRO 2000, both in solution in PBS and in the 4% gel formulation, provided significant protection against disease and infection, compared with the appropriate control group ($P < .001$ each). All 3 PRO 2000–treated animals that became infected were in the group that received the 10% PRO 2000 solution. Consequently, we chose to use the gel formulation in subsequent studies, to further evaluate PRO 2000 as a topical microbicide. In a second study (table 1; series 2), animals treated with the 4% gel 20 s before viral challenge were completely protected. In addition, treatment with a lower dose (0.5%) PRO 2000 gel was also effective, providing substantial but not complete protection against both disease and infection, compared with the placebo gel ($P < .001$ each).

We next examined the effect of the time of application on efficacy. Table 2 shows that mice treated with the 4% PRO 2000 gel 5 min before viral challenge were completely protected against infection. When the gel was administered 15 min before challenge, 11 of 12 mice were protected, and even when the gel was applied 60 min before challenge we observed both significant protection against infection ($P < .01$) and development of disease ($P < .05$).

Discussion

An effective microbicide must be both highly protective and safe even when administered one or more times daily. This safety requirement has led to concerns that frequent application of microbicides containing surface-active agents at concentrations sufficient to inactivate pathogens might also cause damage to the vaginal epithelium and thus, under certain circumstances, actually increase susceptibility to STD infection. Clinical studies with N-9 appear to confirm these concerns, since there is

Table 2. Effect of time of administration on the efficacy of PRO 2000 gel against genital herpes simplex virus type 2 in mice.

Treatment	Time administered[a]	No. inoculated	No. protected against infection[b] (%)	No. protected against disease (%)
Placebo	−20 s	15	1 (7)	4 (27)
4% PRO 2000	−5 min	12	12 (100)[b,c]	12 (100)[c]
4% PRO 2000	−15 min	12	11 (92)[c]	11 (92)[d]
4% PRO 2000	−30 min	12	7 (58)[d]	9 (75)[c]
4% PRO 2000	−60 min	12	7 (58)[d]	9 (75)[c]

NOTE. All treatment was given in gel.

[a] Time relative to virus inoculation.

[b] Animals that did not develop symptoms were defined as infected if virus was isolated from vaginal swabs collected on day 2 after inoculation.

[c] $P < .001$ vs. placebo, Fisher's exact test.

[d] $P < .01$ vs. placebo, Fisher's exact test.

[e] $P < .05$ vs. placebo, Fisher's exact test.

substantial evidence that frequent use can cause inflammation and disruption of the vaginal and cervical epithelium [11, 12]. In addition, while N-9 protected against a number of STD pathogens in animal studies [13, 14], results from clinical trials have been contradictory [4–6]. Thus, there is considerable interest both in the identification of novel compounds with potential as topical microbicides [14–16] and in the development of microbicides, such as PRO 2000, that act by blocking infection rather than by the destruction of the pathogen. It is hoped that such agents, in addition to being highly protective, will not cause cytotoxicity even after frequent use. The results of two recent phase 1 clinical trials provide preliminary evidence that vaginal gels containing ≤4% PRO 2000 are both safe and well tolerated (unpublished data). The results reported here provide the first evidence that PRO 2000 can provide in vivo protection against a recognized STD pathogen, HSV-2.

We show that a single prophylactic application of PRO 2000, either in solution or formulated in a vaginal gel at concentrations used in phase 1 clinical trials, is sufficient to provide significant protection against HSV-2 infection in a mouse model. The 4% vaginal gel formulation was 100% protective when administered shortly before viral challenge and retained good efficacy for at least 60 min. This ability to be effective quickly after application and to maintain protective efficacy for an extended period in the genital tract is an important characteristic for a microbicide, since it is likely that there will be considerable variations between the time of application and exposure to an STD pathogen. On the basis of the observed in vivo effectiveness of PRO 2000 against genital HSV-2 infection, further studies of PRO 2000's potential as a microbicide are warranted.

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